A COMPARATIVE ANALYSIS OF THE DEMOGRAPHIC AND PATHOPHYSIOLOGICAL FACTORS INFLUENCING CELLULAR IMMUNITY IN CHRONIC OTITIS MEDIA-PRONE CHILDREN

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Abstract

Otitis media (OM) is a childhood illness that is caused when viral and bacterial commensals ascend the Eustacian tube to enter the middle ear space, where they cause infection, pain, inflammation, and possible effusion. Host determinants including anatomy, physiology, demography and immunity; microbial factors of nasopharyngeal colonisation, inter-microbial and microbial-host relationships; and environmental factors all impact on the risk of developing OM, and the burden caused by the disease. How each of these factors contributes to the disease aetiology is well defined, yet the relationships between these factors and how such relationships contribute to OM are less clearly understood. Host tolerance to microbial colonisation at mucosal sites, including *Streptococcus pneumoniae* colonisation of the nasopharynx, has been associated with regulatory T (T_{reg}) lymphocytes and suppression of pro-inflammatory responses. What is not known, however, is the association of the T_{reg} lymphocyte population with *Moraxella catarrhalis* and non-typeable *Haemophilus influenzae* (NTHi), or with other otopathogen commensal colonisation at the nasopharynx. Nationally, OM in children from regional Queensland on the eastern coast of Australia has not been investigated until now. Herein, a study cohort of 40 children between 2 and 7 years of age from regional Queensland, who were either chronic OM (COM) prone or non-COM prone were included. For each child, the study has assessed multiple factors of demographic, environmental, nasopharyngeal bacterial carriage, lymphocyte subset proportions from the adenoids and blood, and salivary and plasma pneumococcal-specific antibody titres.

A participant questionnaire was used to gather information on the household environment and family history of OM relevant to the participating children. The participant's clinical history was collected and during scheduled adenoidectomy for clinical reasons, the participant's adenoids, a small peripheral blood sample, a saliva sample, and a nasopharyngeal aspirate (NPA) were each collected for either microbiological, lymphocyte subset proportions or pneumococcal-specific antibody assessments. In order to determine the relationships between the demographic, environmental, microbiological and immunological factors and relate these to a child's susceptibility to COM or upper respiratory tract infections (URTI), or to a child's nasopharyngeal bacterial carriage, during the statistical analysis the study population was split into cohorts of COM prone and non-

COM prone, URTI prone and non-URTI prone, and groups of specific bacterial positive and negative culture.

It was found that of all demographic, environmental, microbiological and immunological factors investigated, none increased the risk of a child's susceptibility to COM or to URTI, and there were no significant differences in these factors between COM prone and non-COM prone children, or between URTI or non-URTI prone children. Among these factors, however, there were significant associations and differences with nasopharyngeal culture in children. The risk of NTHi nasopharyngeal carriage increased significantly in children who were the youngest among siblings, whereas these children had a reduced risk of *Staphylococcus aureus* positive nasopharyngeal carriage. Environmental tobacco smoke (ETS) exposure was shown to increase significantly the risk of *M. catarrhalis* and *S. aureus* nasopharyngeal carriage, and male children had significantly more nasopharyngeal positive culture compared to female children.

NPA cultures of *S. pneumoniae* were found to significantly predict *S. pneumoniae* colonisation at the adenoids. This provides evidence for physicians to potentially use NPA cultures as a novel screening method to determine *S. pneumoniae* colonisation at the adenoids, thereby enabling targeted antibiotic treatment to reduce *S. pneumoniae* carriage in children, while also lessening the number of inappropriate prescriptions of antibiotics.

The presence of circulating $CD19^+$ lymphocytes had a significant, positive association with *M. catarrhalis*, while $CD3^+CD8^+$ lymphocytes from the adenoids had a significant, negative association with *S. aureus*. Lymphocyte subset proportions from the adenoids and blood were not significantly different between NTHi or *S. pneumoniae* culture positive or negative children. However, children with positive nasopharyngeal culture did have significantly increased percentages of circulating T_{reg} lymphocytes, compared to children with negative nasopharyngeal culture. This supports the hypothesis, in regard to systemic immunity but not locally in the adenoids, that commensal bacteria within the nasopharynx are positively associated with T_{reg} lymphocytes in the adenoids and blood of children with COM, thereby potentially contributing to host tolerance of nasopharyngeal colonisation and the development of COM. This is the first report of lymphocyte proportional changes and associations with general nasopharyngeal otopathogen culture, *M. catarrhalis*, *S. aureus* and NTHi culture in children.

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Certificate of Authorship and Originality (Declaration)

I, the undersigned author, declare that all of the research and discussion presented in this thesis is original work performed by the author, except where due reference is made in-text. No content of this thesis has been submitted or considered either in whole or in part, at any tertiary institute or university for a degree or any other category of award. I also declare that any material presented in this thesis performed by another person or institute has been referenced and listed in the reference section at the end of this thesis. In the case of published papers that have been reproduced *verbatim* (intact as published), references are provided at the end of each paper (see Chapter 2). The contributions by others have been acknowledged in the relevant chapters where appropriate, and detailed in the 'Statement of Contributions by Others'.

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Statement of Contribution by Others

The contribution by others to this thesis in providing specialist services and support are listed herein. The level of their contributions is noted.

I would like to acknowledge and thank the participants and their parents for their contribution through the donation of their adenoids, saliva, blood and nasal aspirate samples and the supporting information within the Participant Questionnaire.

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All work undertaken in this study was performed in accordance with the approval of the Human Research Ethics Committee (HREC) of the Mater Hospital Rockhampton, Australia, and of CQUniversity, and was conducted in accordance with the Code of Conduct for Research at CQUniversity.

My supervisors, Professor Jennelle M. Kyd, Professor Andrew W. Taylor-Robinson and Dr Evan H. Matthews supervised my work and provided guidance and assistance. The three supervisor's contributions are acknowledged through co-authorship of conference abstract proceedings and publications arising from this thesis.

Excepting from above, all work was performed, and all the HREC applications, manuscripts and chapters were written, by the undersigned candidate.

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Publications Arising

Published papers

Browne, JJ, Matthews, EH, Kyd, JM & Taylor-Robinson, AW 2013, 'The balancing act between colonisers and inflammation: T regulatory and T_H17 cells in mucosal immunity during otitis media', *Current Immunology Reviews*, vol. 9, no. 2, pp. 57-71, http://www.eurekaselect.com/112910/article (This manuscript comprises Chapter 2 of the thesis).

Manuscripts in preparation

Browne, JJ, Matthews, EH, Taylor-Robinson, AW, Kyd, JM 2016, 'Comparative analysis of demographic factors with infection and immunity in chronic otitis media'. (This manuscript relates to Chapter 4 of the thesis).

Browne, JJ, Matthews, EH, Taylor-Robinson, AW, Kyd, JM 2016, 'Trends in the nasopharyngeal microbiology evaluated with clinical factors of chronic otitis media in children from rural Australia'. (This manuscript relates to Chapter 5 of the thesis).

Browne, JJ, Matthews, EH, Taylor-Robinson, AW, Kyd, JM 2016, 'Adenoid and peripheral blood lymphocyte associations with clinical factors of chronic otitis media in children from rural Australia'. (This manuscript relates to Chapter 6 of the thesis).

Conference Presentations

International oral presentations

Browne, J, Matthews, E, Taylor-Robinson, A & Kyd, J 2015, 'Lymphocytes associated with adaptive immunity from the adenoids and peripheral blood of children from rural Australia and the correlation with chronic otitis media or adenoid hypertrophy', *International Society for Otitis Media - 18th International Symposium on Recent Advances in Otitis Media*, National Harbor, Maryland, June 7-11. Abstract OM2015249.

Kyd, J, **Browne, J**, Krishnamurthy, K & Matthews, E 2015, 'Polymicrobial colonisation associated with chronic otitis media correlates with microbial conditions in vitro that increase adherence, biofilm formation and reduced pro-inflammatory responses with respiratory epithelial cells', *International Society for Otitis Media - 18th International Symposium on Recent Advances in Otitis Media*, National Harbor, Maryland, June 7-11. Abstract OM2015249.

Browne, J, Kyd, J, Taylor-Robinson, A & Matthews, E 2014, 'Cellular immunity in adenoids and blood of otitis media-prone children', *The Australian Society of Otolaryngology Head and Neck Surgery – Indigenous Ear Disease – Closing the Air-bone Gap; satellite meeting to the ASOHNS 2014 Annual Scientific Meeting*, Brisbane, March 28-April 1.

National oral presentations

Browne, JJ, Matthews, E, Taylor-Robinson, A & Kyd JM 2014, 'Cellular immunity in adenoids and blood of otitis media-prone children', *OMOZ 2014 – 3rd Australian Otitis Media Conference 2014*, Melbourne, August 6-8.

List of Abbreviations

AdMNC	Adenoid mononuclear cell
AF647	Alexa Fluor 647
AH	Adenoid hypertrophy
ANOVA	Analysis of variance
AOM	Acute otitis media
APC	Antigen presenting cell
ATSI	Aboriginal and Torres Strait Islander
BgaA	β-galactosidase
BM-DC	Bone marrow-derived dendritic cell
BV421	BD Horizon Brilliant Violet 421
CbpA	Choline-binding protein A
CCMI	Capricornia Centre for Mucosal Immunology
CD	Cluster of differentiation
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CF	Cystic Fibrosis
CFU	Colony forming unit
ChoP	Phosphorylcholine
CI	Confidence interval
CO_2	Carbon dioxide
COM	Chronic Otitis media
COME	Chronic Otitis media with effusion
COPD	Chronic obstructive pulmonary disease
CSOM	Chronic suppurative otitis media
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intracellular adhesion molecule 3
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dPBS	Delbecco's phosphate buffered saline
EC	Epithelial cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

ENT	Ear, Nose and Throat
ETS	Environmental tobacco smoke
FISH	Fluorescent *in situ* hybridisation
FITC	Fluorescein isothiocyanate
FMO	Fluorescent minus one
FoxP3	Fork-head box P3
GALT	Gut associated lymphoid tissue
Hag	*Moraxella catarrhalis* Immunoglobulin D binding protein
HI-FCS	Heat-inactivated fetal calf serum
HMW	High molecular weight
HPV	Human papillomavirus
HREC	Human Research Ethics Committee
HRP	Horse radish peroxidase
HRZN V500	BD Horizon V500
Hyl	Hyaluronate lyase
ICAM1	Intercellular adhesion molecule 1
IFN	Interferon
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
iT_{reg}	Inducible T regulatory cell
KC	Chemokine ligand 1
LC	Langerhans cell
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
M	Mean
McaP	*Moraxella catarrhalis* adhesin protein
MEE	Middle ear effusion
MEF	Middle ear fluid
MEM	Middle ear mucosa
MHC	Major histocompatibility complex
MID	Moraxella catarrhalis IgD binding protein
MIP	Macrophage inhibitory protein

MNC	Mononuclear cell
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
NaCl	Sodium chloride
NALP3	NACHT, LLR and PYD domain-containing protein 3
NALT	Nasal-associated lymphoid tissue
NanA	Neuraminidase
NF-κβ	Nuclear factor-κβ
NK	Natural killer cells
NODs	nucleotide-binding oligomerisation domain-containing proteins
NPA	Nasopharyngeal aspirate
NTHi	Non-typeable *Haemophilus influenzae*
nT_{reg}	Naturally occurring T regulatory cells
oLC	Oral Langerhans cells
OM	Otitis media
OME	Otitis media with effusion
OMP	Outer membrane protein
OMP CD	Outer membrane protein CD
OMP E	Outer membrane protein E
OMV	Outer membrane vesicle
OR	Odds ratios
PAF	Platelet-activating factor
PAFr	Platelet-activating factor receptor
PAMP	Pathogen-associated molecular pattern
PavA	Pneumococcal adhesion and virulence A
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PCV-7	7-valent pneumococcal conjugate vaccine
PCV-13	13-valent pneumococcal conjugate vaccine
pDC	Plasmacytoid dendritic cell
PE-CF594	Phycoerythrin-CF594
PE-Cy7	Phycoerythrin-cyanine dye C7
PerCP-Cy5.5	PerCP-cyanine dye Cy5.5

PHiD-CV	Pneumococcal NTHi protein D conjugate vaccine
PIgA	Plasma immunoglobulin A
PIgG	Plasma immunoglobulin G
pIgR	Polymeric immunoglobulin receptor
PLA	Phospholipase A
PLC	Participant Laboratory code
Ply	Pneumolysin
PRR	Pattern recognition receptor
PSA	Polysaccharide A
PspA	Pneumococcal surface protein A
QUT	Queensland University of Technology
RA	Retinoic acid
rAOM	Recurrent acute Otitis media
rOM	Recurrent otitis media
RSV	Respiratory Syncitial Virus
RV	Rhinovirus
SD	Standard deviation
SIgA	Salivary immunoglobulin A
StrH	β-N-acetylglucosaminidase
T_C	Cytotoxic T lymphocyte
TCR	T cell receptor
T_{FH}	T follicular helper lymphocyte
TFP	Type IV pili
TGF-β	Transforming growth factor-β
T_H	T helper lymphocyte
T_H1	T helper 1 lymphocytes
T_H17	T helper 17 lymphocytes
T_H3	Type 3 inducible T regulatory cell
TLR	Toll-like receptor
TM	Tympanic membrane
TMB	Tetramethyl benzidine
TNF	Tumour necrosis factor
Tr1	Type 1 inducible T regulatory cell

T_{reg}	T regulatory cell
URT	Upper respiratory tract
URTI	Upper respiratory tract infection
UspA1/UspA2	Ubiquitous surface protein A1 and A2
WCSA	Whole cell sonicate antigen
WKC	Whole killed cell

1 INTRODUCTION AND FRAMING

1.1 Introduction to OM, the Study Locale and the Study Outline

In Australia and other developed countries, doctor visits, and analgesic and antibiotic treatments for children are most frequently due to OM, while it is also the second most common cause for child hospitalisation associated with a procedure (Klein 2001; Kong & Coates 2009). Middle ear disease may present in an array of pathologies, although most commonly acute OM (AOM) or chronic OM with effusion (COME) are observed (Leach & Morris 2007). OM is a multifactorial disease caused by viral or bacterial commensals that ascend the Eustacian tube and enter the middle ear cavity, causing infection, inflammation, fluid accumulation, primary otalgia and possible otorrhea. Determinants relating to the microbes, host and the environment also increase the risk and burden of disease (Klein 2001). These include anatomical and physiological factors, demographic and environmental factors, nasopharyngeal colonisation, inter-microbial and microbial-host relationships, and host immune responses. Globally, there is a considerable understanding of how each of these factors contributes to the disease aetiology, yet the relationships among these factors and how such relationships contribute to OM are less clearly defined.

Host tolerance of microbial colonisation at mucosal sites, including colonisation by *S. pneumoniae* of the nasopharynx, has been associated with T_{reg} lymphocytes and suppression of pro-inflammatory responses (Zhang et al. 2011; Palomares et al. 2012; Jiang et al. 2015). The nature and extent of the T_{reg} lymphocyte associations with *M. catarrhalis* and NTHi, or with other otopathogen commensal colonisation at the nasopharynx, are, however, unknown. Across the country, much of the published studies of OM from Western Australia and the Northern Territory relates to Indigenous Australian Aboriginal children who commonly experience a greater disease burden, compared to non-Indigenous Australian children (Leach 1999; Morris et al. 2005; Leach & Morris 2007; Morris et al. 2007; Jacoby et al. 2008; Lehmann et al. 2008; Kong & Coates 2009; Jacoby et al. 2011; Wiertsema et al. 2011). OM in children from the eastern coast of Australia has not been investigated until now. The present study has assessed factors of demographic, environmental, nasopharyngeal bacterial carriage, lymphocyte subset proportions from the adenoids and blood, and salivary and plasma pneumococcal-specific antibody titres in 40 children between 2 and 7 years of age from the Rockhampton area of regional Queensland,

Australia (see Figure 1), who were prone or non-prone to COM as identified upon consultant examination by an Ear, Nose and Throat (ENT) physician.

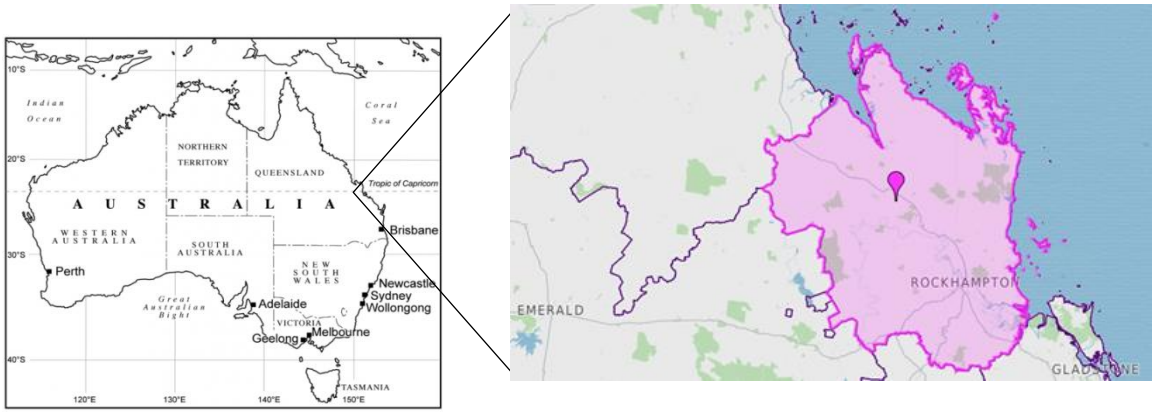

Figure 1 The Rockhampton area of regional Queensland, Australia.
Adapted from Forrest et al. (2006) and the Australian Bureau of Statistics (2014a).

Parents or legal guardians of participants completed a participant questionnaire (see Appendix A) to gather information on the household environment and family history of OM relevant to the participating children, including Aboriginal and Torres Strait Islander (ATSI) heritage; the number of children in the household under 15 years of age; birth order; sibling history of OM; exposure to ETS; attendance at day care, kindergarten, preschool or school; and routine immunisation compliance. Clinical data were collected from the participants' clinical records and all biological samples were collected by an ENT physician during scheduled adenoidectomy for clinical reasons. From each child the adenoids were collected for microbiological and lymphocyte assessment. A small peripheral blood sample was collected for antibody and lymphocyte assessment. A saliva sample was collected for antibody assessment, and a nasopharyngeal aspirate was collected for microbiological assessment. In order to determine the relationships between these factors and to relate these to a child's susceptibility to COM or URTI, or to a child's nasopharyngeal bacterial carriage, during the statistical analysis the study population was split into cohorts of COM prone and non-COM prone, URTI prone and non-URTI prone, and groups of specific bacterial positive and negative culture.

This study provides information required for a better understanding of factors contributing to a child's susceptibility to COM in regional Queensland, on the east coast of Australia. Cellular immune tolerance to nasopharyngeal flora and the relationship with proneness to

COM is not understood, yet there is evidence warranting investigation into the role of T_{reg} lymphocytes with otopathogen colonisation and COM (Zhang et al. 2011; Palomares et al. 2012; Hirano et al. 2015). The Rockhampton area of regional Queensland provided an optimal platform to support this research due to human and research resources established in the region, and collaborations developed with the Mater Hospital Rockhampton, Sullivan Nicolaides Pathology, Rockhampton and partners at the Institute of Health and Biomedical Innovation at the QUT in Brisbane. It was a rare opportunity to provide insight into what COM looks like in Queensland, as it was the first study of its kind in the state. Although it was not expected for OM risk factors to be different from those identified in Western Australia and the Northern Territory, it was a requirement to identify OM risk factors in this study cohort in order to determine risk factors associated with COM proneness in the study cohort and to meet primary outcomes where risk factors were correlated with lymphocyte populations to determine relationships present, if any. Furthermore, when considered with similar studies conducted in Western Australia, the Northern Territory, and New Zealand, this research collectively enables a greater understanding of OM in Australasian children (Leach et al. 1994; Morris et al. 2005; Jacoby et al. 2007; Morris et al. 2007; Jacoby et al. 2008; Jacoby et al. 2011; Wiertsema et al. 2011; Mills et al. 2015).

While this study's main objective was to investigate cellular immune proportions, especially T_{reg} lymphocytes in COM prone and non-COM prone children, and their associations with demographic, clinical and microbiological factors of the participants in the study, it provides further assessment of OM in regional Queensland; a valuable contribution considering the lack of OM research in Queensland generally. The demographic, environmental, microbiological and immunological factors that contribute to a child being prone to COM in regional Queensland are determined by studies done elsewhere, therefore an OM clinical study in Queensland will help assess if risk factors are consistent with other regions. Furthermore, the extent of the relationships between these factors and how these contribute to a child being prone to COM also requires further investigation. This information will contribute to the effective management of COM in children from regional Queensland, by identifying possible risk factors, dominant nasopharyngeal bacterial colonisers and potential microbial screening methods used for targeting antibiotic therapies, thereby reducing the over-prescription of inappropriate antibiotics. Moreover, an understanding of the relationships among nasopharyngeal

otopathogens with local and systemic lymphocyte subset proportions, particularly the T_{reg} lymphocytes, will provide evidence for further investigation into functional aspects of colonisation, tolerance and host immunity, and how these may contribute to a child's susceptibility to COM.

1.2 Aims and Objectives

1.2.1 Primary aims

The primary aims were to:

- Identify and characterise the distribution of B, T, T helper (T_H), cytotoxic (T_C) and T_{reg} lymphocytes as a proportion of the total lymphocyte population in adenoidal and blood tissue of COM prone and non-COM prone children;
- Analyse the B, T, T_H, T_C and T_{reg} lymphocyte populations and to correlate each with the demographic, clinical, and microbiological factors of the participants in the study;
- Determine the relationships between the demographic, environmental, clinical microbiological and immunological factors that contribute to the development of COM in children.

1.2.2 Secondary aims

The secondary aims were to:

- Identify in children within the Rockhampton area of regional Queensland, risk factors associated with proneness to COM;
- Understand how these risk factors relate to contributors of infection and immunity in children prone to COM, with the aim to identify effective intervention strategies;
- Identify important otopathogens in children of regional Queensland prone to COM and URTI;
- Evaluate the potential of using microbial cultures of nasopharyngeal aspirates to predict bacterial colonisation in the greater nasopharynx, for its application as a screening method for clinicians in patient diagnoses;

- Determine if there are differences in the local and systemic lymphocyte populations in relation to adenoid hypertrophy (AH), URTI, nasopharyngeal colonisation and COM in children of regional Queensland;
- Understand how AH, URTI, nasopharyngeal colonisation and COM may influence the distribution of lymphocyte populations, in order to further understand adaptive immune functions relating to clinical factors associated with COM.

1.3 Hypothesis

Commensal bacteria within the nasopharynx are positively associated with T_{reg} lymphocytes in the adenoids and blood of children with COM, thereby potentially contributing to host-tolerance of nasopharyngeal colonisation and to the development of COM.

1.4 Thesis structure

The initial review of literature for this thesis was published as a review article during the period of candidature. Consequently, the thesis is presented in part-publication style wherein a large portion of the literature review is presented as the published peer-reviewed paper. This is reported as *verbatim* in Chapter 2 in the journal's required style and formatting, with its own reference list. The main reference list that comprises Chapter 8 lists all other references cited throughout the thesis. Other materials that provide additional details relevant to concepts supporting the study are presented as supplementary information in Chapter 2. As this study was based on a series of biological sample and data collections, laboratory-based activities and epidemiology-based analyses, Chapter 3 details the study design and methodology, while Chapters 4 to 7 inclusive present and discuss the data and applied analyses. This is in accordance with CQUniversity's current operating policy of 'Publication of research higher degree research work for inclusion in the thesis procedures'.

In Chapter 1, a general introduction to the thesis topic is presented, with concepts and study design discussed. The study hypothesis and aims are outlined, and the thesis structure is explained. Chapter 2 provides a detailed review of the scholarly literature, presenting concepts from published works in the areas of OM, nasopharyngeal bacterial colonisation, immune tolerance to colonisation at mucosal sites, and polymicrobial interactions in the

main discipline areas of microbiology, immunology and epidemiology. Chapter 3 outlines the study design and the methods used in participant recruitment, biological sample and data collection, microbiological assessment, lymphocyte subset analysis, antibody titre measurements and statistical analyses. The methods outlined in Chapter 3 support the data presented and discussed in Chapters 4 to 7.

Chapter 4 examines in detail the associations of the demographic and environmental characteristics with the clinical, microbiological and immunological data sets of the study population, and between the study cohorts of COM prone and non-COM prone children, and URTI prone and non-URTI prone children. Chapter 5 provides a detailed microbiological analysis in which the bacteriology is examined from the NPA and adenoid biopsy samples. Presented are the nasopharyngeal otopathogen distributions within the general study population, and between COM prone and non-COM prone, and between URTI prone and non-URTI prone children. The otopathogen co-colonisation trends, the clinical, demographic and environmental determinants of colonisation, and the relevant bacterial associations between the two nasopharyngeal sites are also examined. Chapter 6 investigates the immunological aspects associated with the study population, including a detailed evaluation of the lymphocyte subset proportions of the adenoids and blood, and the pneumococcal-specific salivary total immunoglobulin (Ig) A (SIgA) and plasma total IgA (PIgA) and IgG (PIgG) titres. Correlations among the local and systemic lymphocyte subsets are discussed, and their distributions in COM prone and non-COM prone, and between URTI prone and non-URTI prone children are presented. The pneumococcal-specific SIgA, PIgA and PIgG titres are also compared between these cohorts, and their correlations with pneumococcal-associated nasopharyngeal culture from the children are determined. A discussion is also provided regarding the correlations of the lymphocyte subset proportions in the adenoids and blood with the nasopharyngeal bacteriology from the children. Chapter 7 provides a detailed summary of the presented collective findings, with suggested areas for further research. Chapter 8 presents a comprehensive list of references cited in the thesis, other than those cited only in the published review.

1.5 Proposed Contribution to the Field

Immune suppression has been identified as a contributing factor in the aetiology of OM (Rynnel-Dagöö & Ågren 2000; Eun et al. 2009). This debilitating disease of the upper

airways affects children across Australia and throughout the world, imposing a significant impact on 80 percent of Australian children by 3 years of age (Kong & Coates 2009). Indigenous Aboriginal children are a high risk population with greater than 90 percent aged between 6 and 30 months experiencing OM. In 45 percent of cases a documented perforation is evident, resulting in poor hearing and speech, and under-developed learning and social behaviour (Morris et al. 2005). This study aims to identify the demographic, environmental, clinical and microbiological factors associated with T_{reg} lymphocytes in the adenoids and blood, providing evidence for how these factors correlate with immune suppressive lymphocytes, and therefore infection tolerance in COM prone children. In identifying what factors correlate with the presence of T_{reg} lymphocytes in the adenoids and blood of COM prone children, this will provide the information required for future studies to investigate if such factors have a causal relationship with a T_{reg} lymphocyte phenotype, and if this induces tolerance to otopathogens, thus promoting chronicity of infection in children. Once such relationships and mechanisms are understood, further research may focus on the manipulation of such immune pathways to promote increased clearance of the upper airway infections and to reduce COM, thereby decreasing the overall incidence and severity of the disease, and complications associated with prolonged clinical manifestations.

2 LITERATURE REVIEW

2.1 Published paper: "The Balancing Act Between Colonisers and Inflammation: T Regulatory and T_H17 Cells in Mucosal Immunity During Otitis Media".

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The Balancing Act Between Colonisers and Inflammation: T Regulatory and T_H17 Cells in Mucosal Immunity During Otitis Media

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Abstract: Inflammation of the middle ear, otitis media, is a significant cause of pain and reduced auditory acuity in children. Recurrent episodes may delay development of speech, learning and social behaviour. *Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae* and *Moraxella catarrhalis* are most often implicated. These bacteria colonise the nasopharynx asymptomatically but host tolerance of high nasopharyngeal load contributes to onset of inflammation. Immunosuppression is evident in susceptible children which may contribute to tolerance and therefore to progression to chronic disease. While the causative factors involved in the immunosuppressive response are not known, evidence from other mucosal sites suggests that T regulatory (T_{reg}) lymphocytes, a subset of T helper (T_H) lymphocytes, contribute to regulation of immunosuppression to commensal bacteria and promote advancement of infection. The major function of T_{reg} lymphocytes is induction of immune tolerance via immunosuppression in the periphery to foreign and self antigen. They have been identified in adenoids and tonsils and are known to have a positive association with pneumococcus nasopharyngeal colonisation. Interestingly, the pro-inflammatory T_H17 lymphocyte response to *S. pneumoniae* is reduced in pneumococcal-positive children. Furthermore, inadequate T lymphocyte proliferation to non-typeable *H. influenzae* is evident in otitis media-prone children. A weak T lymphocyte repertoire in young children may explain high nasopharyngeal bacterial carriage observed in this population. However, T_H17 and T_H1 lymphocyte responses may be subdued due to T_{reg} lymphocyte suppression. The immune factors that regulate nasopharyngeal colonisation are not well understood and further research is required to elucidate the immunological mechanism that underlies development of otitis media.

Keywords: Commensal, immunity, mucosal, nasopharynx, otitis media, T regulatory lymphocytes.

INTRODUCTION

"Inasmuch as the nasopharyngeal tonsil is the critical site for the early events that will lead to the development of both otitis media and sinusitis, it appears that manipulation of this area with strategies other than antibiotics could be successful in the prevention of colonization and the subsequent development of inflammation of the upper respiratory tract" [1]. This statement provides the critical reasoning for the importance of investigating characteristics of the adenoid that may contribute to the pathogenesis and persistence of otitis media (OM), inflammation and dysfunction of the middle ear. For centuries, dating back as far as Hippocrates in 400 BC, OM has been documented, yet approximately 2500 years on OM remains a prevalent disease within a modern society, but it has evolved into a well-defined condition [2]. The aetiology of OM is complex and may be attributed to multiple factors including age, viral and bacterial milieu, congenital or acquired immuno-deficiency, allergy, Eustachian tube dysfunction or facial structure abnormalities, genetic, racial, socio-economic and environmental exposures [3].

OM is a major burden on health services worldwide. Native American, Alaskan, Canadian and Australian Aboriginals are ethnic populations at high risk for developing OM [4-6]. More than 80% of Australian children will have suffered with an OM infection by age three. Of these, almost 40% of children will develop recurring infections, with little relief experienced from antibiotic therapies [7]. Children suffering with these infections experience pain and decreased hearing that may be acute or chronic, with long-term consequences including poor hearing and speech, and underdeveloped learning and social behaviours [5]. The current estimated burden of OM on the Australian Health Services exceeds $100 million annually and is due largely to a lack of available preventative therapies [7].

It has become evident through rigorous clinical research that immunosuppression is evident in OM-prone children, which may contribute to the pathogenesis of OM and the progression to chronic disease [8, 9]. What determines these children to have an immunosuppressive response is still unclear. However, there is evidence at other mucosal sites under similar microbial loads that T regulatory (T_{reg})

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lymphocytes, a subset of T helper (T_H) lymphocytes, contribute to the regulation of immunosuppression to commensal bacteria and contribute to the progression of infection and chronic disease [10]. The adenoids and tonsils are the only secondary lymphoid organs that are localised to the nasopharynx and middle ear. There is evidence that adenoidectomy in children suffering from chronic OM improves the clinical outcome by reducing the incidence of disease [1]. It is often suggested that these improvements are due to the removal of the inflamed, and sometimes necrotic, tissue and the associated microbiological reservoir [11]. It remains unknown, however, whether or not this phenomenon occurs due to factors associated with local cellular immunity due to removal of the secondary lymphoid organs of the nasopharynx.

This paper presents the microbiology and pathophysiology of OM, with an overview of the current understanding of cellular immune factors associated with OM. The T_{reg} and T_H17 cellular populations are a particular focus, with discussion of their importance in balancing the inflammatory response to common commensals and opportunistic pathogens at mucosal sites.

THE RESPIRATORY SYSTEM

The three major compartments of the respiratory system include the lower respiratory tract, nasopharynx and middle ear (Fig. **1**). These three sites of the respiratory system are vulnerable to many infections, as the nasopharynx is host to a milieu of viral and bacterial organisms [12]. The epithelium, mucous secretions, ciliary clearance and various immunological cells are important physical, cellular and chemical defences the body employs to clear the respiratory tract of foreign particles and harmful microbes. When these defences become compromised, infection can occur, causing discomfort, pain and impaired function [13]. Although it is evident that the physiology of the Eustachian tube influences the development of middle ear infections, what is not well understood is what immune factors mediate the progression from nasopharyngeal colonisation to middle ear infections [14].

Immunocytology and Physiology of the Nasopharyngeal Lymphoid Tissue

The secondary lymphoid organs in the upper respiratory tract (URT) are localised in the Waldeyer's ring. This is an arrangement of four secondary lymphoid organs in a circular rotation of the throat that consists of the palatine, tubal, lingual and nasopharyngeal (adenoid) tonsils [15]. The palatine and lingual tonsil and the adenoid are the dominant lymphoid organs of the Waldeyer's ring with the palatine tonsil and adenoid most studied due to their availability from tonsillectomy and adenoidectomies [16]. Although the tonsils and adenoids have a complex physiology, two regions of interest are the extrafollicular areas and mantle zones as this is where cellular and humoral acquired immunity is most active within these tissues. In the extrafollicular areas, dendritic cells (DC), interdigitating DC, macrophages, mast cells and lymphocytes are found [16-19]. Within the lymph nodes of the adenoids and tonsils are the follicular zones and the germinal centres (Fig. **2**). Follicular DC with long processes extending out to lymphocytes and plasma cells, and mast cells are found in the lymph nodes. However, the

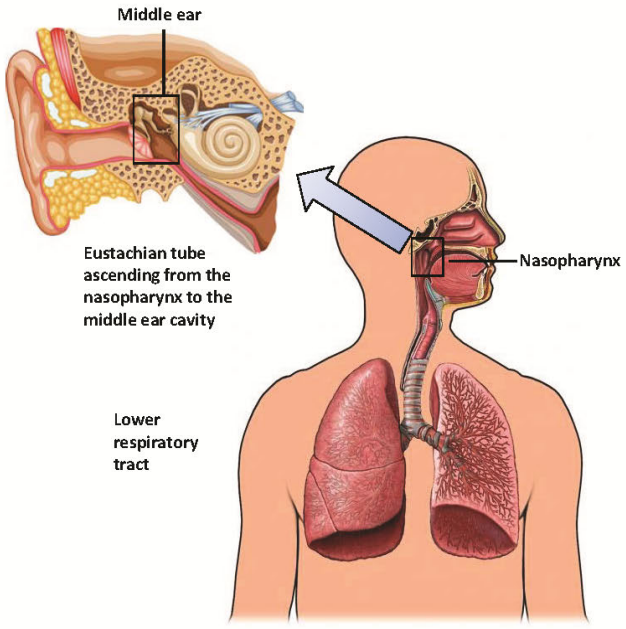

Fig. (1). The respiratory system showing the anatomy of the upper respiratory tract.

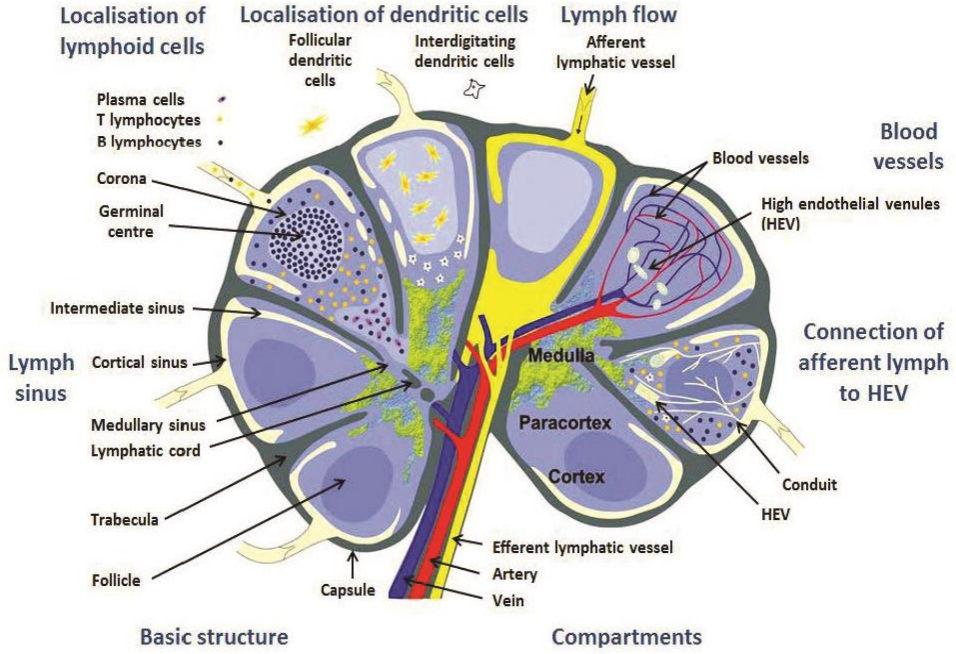

Fig. (2). Schematic view of the substructure of adenoid lymphoid tissue. The cortex contains mostly B lymphocytes organised as primary or secondary follicles. Migration of these cells towards the follicles is mediated by follicular dendritic cells also located in the cortex. T lymphocytes migrate to the neighbouring region, the paracortex, where they interact with interdigitating dendritic cells. The central region, the medulla, consists mainly of B lymphocytes and plasma cells. Lymphocytes enter the lymph node via the afferent lymphatic vessel or through transmigration of high endothelial venules. Lymph and blood vasculatures are connected via a conduit system and both drain into the efferent lymph vessel via the medullary sinus. Redrawn from [138].

follicular zones consist mainly of T lymphocytes while the germinal centres are abundant in naive B lymphocytes [17, 20]. As the adenoids and tonsils are the primary source of immune cells in the upper airways, it is speculated that local immune regulation of the middle ear and nasopharynx may come from these secondary lymphoid organs, although this concept is very under-researched and lacks understanding [1].

Immune Factors of the Nasopharynx and Middle Ear

Immune system factors present in the nasopharynx include interferon (IFN) types I and III, β-defensins, lactoferrin, lysozyme, cathelicidins and mucins, all of which have antimicrobial properties, although the IFNs and cathelicidins are yet to be identified in the human middle ear or nasopharyngeal lymphoid tissue [13, 21-31]. Down-regulation of mRNA or protein expression of the microbial molecule-specific pattern recognition receptors (PRR) retinoic acid-inducible gene 1, NACHT, LRR and PYD domains-containing protein 3 (NALP3), and Toll-like receptors (TLR) 3, 4, 7 and 9 is evident in the accumulation of fluid (effusion) in the middle ear and in the middle ear mucosa of OM-prone children compared to non OM-prone children [32, 33]. Lymphoepithelial tissue of the tonsil and adenoid express TLR4, 7, 9 and, especially strongly, TLR3, which is a significant PRR for antiviral responses [34, 35]. Additionally, a large number of IFN inducible genes and signal and regulatory factors are up-regulated in human middle ear epithelial cell (EC) cultures in a dose- and time-dependent manner in response to Influenzae A virus infection [36]. These include myeloid differentiation primary response gene 88 (MyD88), a signal transducing adaptor protein used by most TLRs to activate the 'rapid-acting' primary transcription factor NF-κB in response to harmful cellular stimuli, and interferon regulatory factors 1 and 7, signalling factors for the production of pro-inflammatory cytokines and IFNs [36]. Other examples include genes that encode for the proteins vipirin, myxovirus resistance 1 and 2, and 2',5'-oligoadenylate synthetase 1 and 2, all of which are involved with degradation of viral components and inhibition of viral replication [36]. Based on the evidence that human middle ear mucosa and nasopharyngeal lymphoid tissue have EC that express PRR, signal and regulatory factors and IFN inducible genes designed for viral detection and inhibition, and that both sites are known for the isolation of common respiratory viruses such as respiratory syncitial virus (RSV) and rhinovirus (RV) that often predispose bacterial OM, it is possible that the human middle ear and the nasopharyngeal lymphoid tissue are

potential sites for type I and III IFN production. This is due to the fact that the above innate immune response molecules are necessary for downstream IFN production following viral pathogen-associated molecular pattern (PAMP) recognition. Supporting this notion are studies in mice which have shown the ability of the nasal-associated lymphoid tissue (NALT) to express IFN-α and IFN-β mRNA and multiple IFN-stimulated genes following pneumococcal colonisation [37]. The detection of IFNs in the middle ear mucosa of rodents has also been reported [38].

TLR and nucleotide-binding oligomerization domain-containing proteins (NODs), also involved in pathogen recognition, are expressed in the middle ear mucosa, although at reduced levels in OM-prone children [33]. The distribution of these PRR between the nasopharynx and middle ear is unknown, although research in rodents has demonstrated that PRR expression increases at the proximal end of the Eustachian tube [39]. This could indicate that the innate immune mechanisms of the nasopharynx have evolved to prevent colonisation at this site in order to maintain the sterile environment of the middle ear and thereby prevent infections. The immune processes in the URT contributing to such host homeostasis and colonisation are, however, poorly understood [39, 40]. TLR, γδ T lymphocytes, intraepithelial lymphocytes, natural killer (NK) cells, DC, T lymphocytes, B lymphocytes, neutrophils and macrophages are also distributed throughout the nasal mucosa [34, 35, 41, 42]. Some of these cell types have been identified in the middle ear mucosa and in middle ear effusion, although they are not as well characterised in this region of the nasopharynx [32, 33, 43, 44]. In animal models of OM it is known that lymphocytes enter the middle ear via blood circulation [45]. However, what remains to be established is how local or distal lymph trafficking influence the acquired response in this site. Furthermore, the immune trafficking of lymphocytes to the middle ear in the human is not understood.

OTITIS MEDIA

OM is defined as inflammation of the middle ear associated with an effusion within the middle ear [46]. OM presents in a range of pathologies from acute OM (AOM) through to chronic suppurative OM (CSOM). Symptoms differ in the degree of severity from mild inflammation to tympanic membrane perforation with effusion [46]. AOM presents with at least one of the acute signs of inflammation of the middle ear such as otalgia, irritability, bulging or redness of the tympanic membrane, otorrhoea or fever. In the early stages of infection middle ear effusion may not be present. However, quite often infection progresses in which middle ear effusion (MEE) accompanies the signs of inflammation and is visible as cloudiness within the middle ear [47]. AOM may progress to OM with effusion (OME) that is defined due to the presence of MEE, although unlike AOM, there are no obvious signs of acute inflammation. Some temporary hearing loss may be evident in both conditions [47, 48]. AOM and OME may evolve to a chronic condition if it is recurrent for more than three times in six months, known as recurrent AOM. If the infection persists for 3-12 weeks it becomes subacute and if longer than 12 weeks it is known as chronic OM (COM) or COM with effusion (COME) [47]. Acute exacerbations may be evident during the course of COM and COME, and this often presents with a purulent discharge or suppuration, hence the term CSOM. Acute suppurative OM may also occur, depending on the severity of the infection [47].

Otitis Media Pathophysiology

OM is a complex polymicrobial disease in which infections occur with virus, bacteria or sometimes both concurrently. The respiratory viruses that cause OM or predispose to the bacterial infections that cause OM include RSV, influenza A and B virus, parainfluenza virus type 1, 2 and 3, adenoviruses, enteroviruses and RV [49]. Clinical investigations have confirmed that the actiology and pathogenesis of OM is associated with upper respiratory viral infections [50]. These infections exacerbate the clinical and bacteriological outcome of OM by compromising mucosal physical barriers, enhancing bacterial adherence to respiratory EC, and altering immune cell function and gene expression [49, 51, 52]. *Streptococcus pyogenes*, group A *Streptococcus*, *Pseudomonas aeruginosa*, *Staphylococcus aureas* and *Alloiococcus otitidis* are bacteria that may cause OM, however not predominantly [53-55]. The three most common species of bacteria cultured from OM infections are non-typeable *Haemophilus influenzae*, *Moraxella catarrhalis* and most commonly cultured, *Streptococcus pneumoniae* [52, 54]. A factor in common to all these bacteria is that they are commensals of the nasopharynx which become aggressive opportunistic pathogens when physiological conditions are compromised and/or there is a shift in immune homeostasis. This may be influenced by carriage load, changes in the nasopharyngeal mucosa integrity, microbial community in the nasopharynx and the associated immune response. All of these factors are intricate and involve multifactorial microbial and immunological processes that are not entirely understood [56-59].

S. pneumoniae, *M. catarrhalis* and non-typeable *H. influenzae* colonise the nasopharynx asymptomatically but host tolerance of their high nasopharyngeal loads contributes to the development of OM. While *M. catarrhalis* naturally colonises healthy children, carriage rates may be as high as 100% by three months of age [60]. Likewise, pneumococcal colonisation establishes early in life and also occurs in high loads, accounting for up to 70% of nasopharyngeal bacterial colonisation in some high risk populations [52, 61]. Non-typeable *H. influenzae* colonisation of the nasopharynx constitutes a large proportion of the *Haemophilus* species that account for around 10% of nasopharyngeal normal flora [62]. Like *M. catarrhalis* and *S. pneumoniae*, non-typeable *H. influenzae* has a dominant nasopharyngeal carriage rate of approximately 60% in infants in some ethnic populations but colonises the nasopharynx after *M. catarrhalis* and *S. pneumoniae*. As the immune system develops, particularly cellular immunity, pneumococcal carriage will decrease to between 2-10% by 10 years of age, and by adulthood *M. catarrhalis* colonisation will occur in only 1-5% of individuals. However, during OM and other respiratory tract infections, nasopharyngeal colonisation of these commensals increases [60, 63]. Higher carriage rates of *M. catarrhalis* may also be evident in persons with pre-existing respiratory conditions including allergic sinusitis and chronic

obstructive pulmonary disease [58, 64]. Collectively, this research indicates a shared characteristic of these bacteria to colonise nasopharyngeal mucosa in high loads during infancy and early childhood, with colonisation levels diminishing into adulthood. Although this may coincide with maturation of immunity, the immunological factors that regulate bacterial colonisation are not well understood and therefore merit a greater focus in current research of OM [52, 65].

THE IMMUNE RESPONSE TO NASOPHARYNGEAL COLONISATION

The polymicrobial features and carriage load variations which have been described are characteristics of nasopharyngeal colonisation that influence immune responses at this site. Nasopharyngeal colonisation studies of *S. pneumoniae* and of *H. influenzae*, in either murine colonisation models or human respiratory epithelial *in vitro* models, demonstrate enhanced local acute inflammatory responses during dual colonisation compared to single colonisation. Neutrophil influx is increased in the nasal mucosa of mice during dual colonisation compared to single colonisation. Furthermore, *S. pneumoniae* nasal colonisation was reduced in the presence of *H. influenzae*, and this was found to be mediated through components of *H. influenzae* activating complement-dependent neutrophil phagocytic killing of *S. pneumoniae* [66]. The inflammatory cytokines involved in neutrophil recruitment, macrophage inhibitory protein (MIP) 2 in mice and interleukin (IL)-8 in humans, are also elevated during dual colonisation [66, 67]. In this rodent model it was found that the MIP 2 induction was dependent on *S. pneumoniae* production of pneumolysin and the activation of the p38 mitogen-activated protein kinase [67]. In single bacteria nasal colonisation rodent *in vivo* and *ex vivo* models, complement-mediated neutrophil phagocytosis has also been shown to have a role in the clearance of *H. influenzae* nasal colonisation [68].

During the inflammatory response, the platelet-activating factor receptor (PAFR) expression is up-regulated on epithelial and endothelial surfaces [69]. *S. pneumoniae* binds to the PAFR through its ligand phosphorylcholine, which upon ligation, *S. pneumoniae* undergoes translocation into the cell via endocytosis [70]. The early inflammatory process associated with this infection state is due largely to neutrophil infiltration into the mucosa from transendothelial migration [71]. A study using a transmigration *in vitro* model has shown that neutrophils migrate across an endothelial monolayer in response to live wild-type *S. pneumoniae* in a dose-dependent manner; however, killed wild-type *S. pneumoniae* and mutant pneumolysin-deficient *S. pneumoniae* only induce neutrophil migration at a minimal level, indicating that the pneumococcal toxin, pneumolysin, and live *S. pneumoniae* are important factors in eliciting a potent early inflammatory response in the mucosa [71]. The PRR TLR2, TLR4 and NOD1 are important in the clearance of encapsulated strains of *H. influenzae*, and neutrophils were found to enhance the killing of *H. influenzae* when accompanied by TLR4 signalling pathways [68]. Mice lacking TLR4 had enhanced nasal colonisation loads of *H. influenzae*, however not as effectively as mice lacking TLR2 or NOD1. The TLR4 knockout mice did, however, exhibit significantly higher levels of *H. influenzae* prolonged survival (colonisation levels detected at 14 days post inoculation) compared to the TLR2 and NOD1 knockout mice. Interestingly, TLR2 knockout mice had diminished neutrophil activation compared to TLR4 and NOD1 knockout mice, suggesting that the TLR2 signalling pathway may be important in controlling encapsulated *H. influenzae* colonisation through neutrophil activation. Taken together, these findings indicate that TLR2, TLR4 and NOD1 signalling pathways are important in the host's innate immunity to nasal colonisation by encapsulated strains of *H. influenzae* [68]. In contrast, the lack of expression of TLR2, TLR4 or NOD1, and a deficiency in neutrophils, did not alter the clearance of non-typeable *H. influenzae* (non-encapsulated strains) indicating that there are redundancies in place that can eliminate this coloniser, likely complement-induced antibody opsonisation-mediated phagocytosis as the lack of the polysaccharide capsule renders the bacteria more susceptible to such immune mechanisms [68]. The signalling molecule MyD88 that is common to the TLR family signalling cascade has been found to be crucial in host immunity to *S. pneumoniae* nasal colonisation and systemic infection [72]. In nasal colonisation and infection rodent models, mice lacking MyD88 had higher *S. pneumoniae* nasal colonisations loads, more severe lower respiratory infections and systemic infections indicated by a quick onset and high bacterial loads, significantly decreased survival rates, and decreased innate immune responses indicated by reduced neutrophil and polymorphonuclear leukocyte lung infiltration, decreased tumour necrosis factor (TNF)-α, IL-6 and chemokine ligand 1 (KC) and reduced signs of an inflammatory reaction in the lungs. These results clearly indicate that MyD88 in the TLR signalling pathway is crucial in the local and systemic cytokine and leukocyte-associated immune response to *S. pneumoniae* colonisation and infection [72].

Cytokine Responses to Nasopharyngeal Colonisation

The middle ear mucosa is capable of mounting an inflammatory response to OM pathogens including *S. pneumoniae* and *A. otitidis*. During such an inflammatory response cytokines including IL-8, IL-1β, IL-6 and TNF-α are generated which represent both innate and adaptive immunity lineages [73]. Children with recurrent OM have demonstrated impaired IL-2 and IL-4 production by adenoidal lymphocytes after restimulation with *S. aureus*. Furthermore, IFN-γ is released by adenoidal lymphocytes in response to the same restimulation. However, this T_H1 response is subdued in such lymphocytes compared to peripheral blood lymphocytes, indicating that mucosal immunity to nasopharyngeal colonisation is suppressed when compared to the systemic response [74]. Mice starting out at 1, 2 and 6 weeks old, representing neonatal, infant and adult mice, respectively, showed significant differences in their macrophage, chemokine and cytokine responses when challenged with *S. pneumoniae* in a 7 or 14 day colonisation model. Overall, the neonatal and infant mice had reduced granulocyte colony-stimulating factor, granulocyte macrophage colony-stimulating factor, monocyte chemotactic protein-1 and the neutrophil attractant chemokine (C-X-C motif) ligand-1. IL-1α, IL-6, TNF-α and IFN-γ cytokines

were also significantly reduced compared to the response of adult mice. The only exception to this trend was the response of IL-10, which was 15-fold higher in neonates than in adult mice [65]. These experimental findings indicate that in infancy, neutrophil recruitment and activation, T lymphocyte development and survival, and induction of T_H1 and T_H17 responses may be impaired as the cytokines and chemokines that are restricted in the infant mouse are important to these innate and adaptive immune processes. This is particularly important in relation to pneumococcal nasal colonisation since neutrophils and T_H17 lymphocytes are regarded as important mediators of *S. pneumoniae* clearance [75].

Adaptive Immunity to Nasopharyngeal Colonisation

The adaptive component of the immune system involves a complex network of many cell types and molecules that act in concert to mount an aggressive, quick and effective response to counter a challenge with a pathogenic microbe. In addition to EC, the key immunological cells include activated myeloid cells, such as macrophages, monocytes and DC, as well as B lymphocytes and a variety of subsets of T lymphocytes of polarised function. During T_H lymphocyte activation the cells will adopt a particular cytokine profile and mature into effector lymphocytes of the phenotype T_H1, T_H2, T_H3, T_H9, T_H17, T_H22 or T follicular helper (T_{FH}) lymphocytes. Through cell to cell contact or messenger molecules such as chemokines and cytokines, these cell types communicate with each other to generate an optimal response to clear an infection [76]. In some cases immune tolerance may be induced for non-threatening commensals at mucosal sites or to suppress excessive inflammatory responses that may be damaging to host tissue [10, 77, 78].

In the URT it remains unclear exactly what adaptive immune pathways and mechanisms regulate the response to bacterial colonisation. The bacterial load that is present at any given time may influence immune function in the nasopharynx as there is a direct correlation between nasopharyngeal bacterial load and increased proliferation of T and B lymphocytes [79]. Since larger lymphocyte populations do not necessarily equate to greater lymphocyte activation, this often contributes to the problem of hypertrophic adenoids in diseased patients [80]. Investigations into the effects of pneumococcal colonisation has revealed that control of pneumococcal carriage may be independent of antibody neutralisation [75]. Recent reports have shown that in mice T_H17 $CD4^+$ T lymphocytes produce the pro-inflammatory cytokine IL-17A to induce a monocyte/macrophage and neutrophil cellular-mediated reduction in pneumococcal colonisation [75, 81]. An increase of IL-17A has also been identified in human tonsillar tissue stimulated with pneumolysin-producing pneumococcal whole cell antigen. *In vitro* studies using human neutrophils demonstrate an 1L-17A dose-dependent neutrophil-mediated killing of *S. pneumoniae* [75]. Pneumococcal carriage in children has also been shown to induce $CD4^+$ T lymphocyte-mediated protective immunity in peripheral blood and adenoidal mononuclear cells. Interestingly, a decrease in the $CD4^+$ T lymphocyte response was evident in pneumococcal culture-positive children compared to children with an absence of pneumococcal nasopharyngeal culture [82]. Collectively, these results support the notion that pneumococcal colonisation is mediated via $CD4^+$ T lymphocytes but that *S. pneumoniae* itself may influence the dynamics of this response. Although non-typeable *H. influenzae* has been demonstrated to activate T and B lymphocytes from tonsillar tissue and to induce a T_H1 lymphocyte response, inadequate T lymphocyte proliferation to the P6 antigen was evident in OM-prone children compared to non OM-prone children [83, 84]. A weak T lymphocyte repertoire in young children may explain the high pneumococcal and non-typeable *H. influenzae* carriage observed in this population, or the T_H17 lymphocyte response may be subdued due to T_{reg} lymphocyte suppression. Further research in this area is needed to elucidate fully the mechanisms involved.

Whereas *S. pneumoniae* and non-typeable *H. influenzae* appear to induce T lymphocyte responses, *M. catarrhalis* may be able to induce lymphocyte responses that are thymus-independent. *M. catarrhalis* immunoglobulin (Ig) D-binding (MID) protein has been demonstrated to induce B lymphocyte proliferation and activation with T_H2 cytokine co-stimulation, in the absence of T lymphocytes. Supplementing the cultures with recombinant CD40 ligand enhanced both the B lymphocyte proliferative response and antibody production, indicating that T lymphocytes may enhance a *M. catarrhalis* MID protein-induced B lymphocyte response. Of note, further activation of T lymphocytes by *M. catarrhalis* MID protein was poor, supporting the view of a B lymphocyte cellular response to *M. catarrhalis* [85].

Antibody Responses to Nasopharyngeal Colonisation

Adenoidal tissue from children with OM has been demonstrated to generate *S. pneumoniae* and *H. influenzae* type b-specific IgG and IgA antibody, with production of IgG dominating over that of IgA. These antibody responses to the two nasopharyngeal colonisers are also more prominent in the adenoidal secretions compared to the peripheral blood, indicating that the pathogen-specific humoral response is compartmentalised in the mucosa [86]. Interestingly, reduced immunity is often evident following respiratory bacterial colonisation, especially in individuals prone to respiratory infection. Poor inflammatory responses have been associated with *M. catarrhalis* colonisation of the luminal regions of the lower respiratory tract [87]. A lack of secretory antibody to *M. catarrhalis* and *S. pneumoniae* outer membrane proteins has been reported in children aged from birth to 2 years who show active nasopharyngeal colonisation of both bacteria. In adults, however, secretion of salivary IgA to multiple outer membrane proteins of *M. catarrhalis* has been demonstrated, indicating that repeated or prolonged exposure enhances the mucosal antibody response [88, 89]. Furthermore, there is a significant reduction in antibody responses to the non-typeable *H. influenzae* P6 antigen in OM-prone children compared to non OM-prone children [90]. Although an underdeveloped immune system in early childhood may contribute to impaired immunity, it does not explain sufficiently why OM-prone children do not mount an adequate Ig response against the nasopharyngeal flora, as demonstrated in non OM-prone children of the same age. Collectively, this may indicate immunosuppression during nasopharyngeal colonisation

with the aforementioned bacteria and hence host tolerance of high carriage loads contributing to the development of OM or other URT infections. Unfortunately, very little is understood of the crosstalk between innate and adaptive immunity in the tolerance of nasopharyngeal flora or how the co-colonisation of many microbes in the nasopharynx may affect immune dynamics [35]. By investigating the role of T_{reg} lymphocytes in URT colonisation and how these cells are induced at this site, a clearer understanding may emerge of host tolerance to nasopharyngeal colonisation and progression to chronic disease.

Mucosal Immunity Versus Systemic Immunity

Although both cellular and humoral immunity are important to the homeostasis of nasopharyngeal bacterial colonisation, there is evidence to suggest that immune responses at mucosal sites are compartmentalised from systemic responses such as those in the peripheral blood [91]. While nasopharyngeal colonisation of *S. pneumoniae* is controlled by a T_H17 $CD4^+$ T lymphocyte response, systemic infections such as bacteraemia are combated via an antibody-mediated opsonisation inducing phagocytosis that is independent of a $CD4^+$ T lymphocyte response [91]. Interestingly, however, pre-nasopharyngeal colonisation of *S. pneumoniae* confers an enhanced antibody-mediated protection to systemic challenge that acts via natural immunity [91]. Recent studies on nasopharyngeal pneumococcal colonisation detected induction of several pneumococcal antigen-specific serum IgG responses in children 12-24 months of age. Unfortunately, this humoral immunity failed to protect against pneumococcal nasopharyngeal recolonisation, albeit due to the polymorphic and capsular shielding nature of *S. pneumoniae* or the shortfall of systemic immunity to confer mucosal protection. Hence, it seems that T lymphocytes of the mucosa are more promising in control of pneumococcal colonisation in the nasopharynx [92].

The OM pathogens *S. pneumoniae*, *M. catarrhalis* and non-typeable *H. influenzae* have been shown to induce cellular responses including the activation of B and T lymphocytes from peripheral blood. Induction of cytokines with a T_H1 lymphocyte signature was also evident, although T lymphocytes were found not to be the cellular source [93]. It is known that these OM pathogens can activate NK cells and that the cytokine profile observed may originate from NK cells in an early innate response that aids the activation of T lymphocytes for a downstream T_H1 lymphocyte cascade [93-95]. Of the little that is known of cellular immunity to OM pathogens, most relates to systemic responses which at best play a very limited role in regulation of bacterial colonisation at the nasopharyngeal mucosal site. It is important to note that microbial challenge at one mucosal site can often confer a certain protection from microbial challenge or hypersensitivity at another mucosal site within the body. This is evident in patients suffering from chronic *Helicobacter pylori* infection of the gastrointestinal mucosa, who demonstrate protection from hypersensitivity disorders such as asthma in the lower respiratory mucosa [96]. In a rodent model of experimentally-induced allergic airway disease, recently this phenomenon has been attributed to *H. pylori*-induced T_{reg} lymphocytes migrating to the lungs and conferring protection to hypersensitivity via the immunosuppressive effects of intrinsic IL-10 production, as evidenced by reduced T_H2 and T_H17 cellular responses (Fig. **3**) [97, 98].

γδ T lymphocytes are a small subset of T lymphocytes that represent approximately 2% of the total T lymphocyte population. These cells differ from conventional T lymphocytes in the structure of their T cell receptor (TCR), switching the conventional α and β chains with one γ and one δ chain. γδ T lymphocytes are not restricted to major histocompatibility complex (MHC) class I or II recognition as they can identify whole proteins without the requirement for these to be processed and presented via antigen-presenting cells (APC). γδ T lymphocytes are found abundantly in the intestinal, nasal and bronchial mucosa where they work in close association with local intraepithelial lymphocytes in the epithelial mucosal layer and are therefore important contributors to mucosal immunity [42, 99]. A role has been reported for γδ T lymphocytes in regulation of pulmonary inflammation in a *S. pneumoniae* infection model [100]. At 7-10 days post *S. pneumoniae* intranasal challenge, clearance of bacteria was evident but lungs remained inflamed. By this time, γδ T lymphocyte populations had infiltrated the lungs significantly, with more than a 30-fold increase compared to naive mice, observed as a localised mucosal response [100]. Numbers of $CD4^+$ and $CD8^+$ T lymphocytes, B lymphocytes and NK lymphocytes were up to double those found in controls, suggesting that due to their dominant presence γδ T lymphocytes have an important role in reducing *S. pneumoniae*-associated pulmonary inflammation [100]. γδ T lymphocyte-deficient mice were equally efficient at clearing *S. pneumoniae* from the lungs compared to wild-type mice. It was determined that γδ T lymphocytes reduce lung inflammation and granuloma formation by inhibiting the alveolar macrophage and pulmonary DC response through direct cytotoxicity [100]. Although this indicates that γδ T lymphocytes are not associated with immunity to *S. pneumoniae*, it does point to their importance for controlling the inflammatory pathology associated with *S. pneumoniae* infections and that this response is limited to the pulmonary mucosa. This highlights how γδ T lymphocytes are instrumental to a controlled local response that avoids compromising immune function at a systemic level.

Antigen Influences T Helper Lymphocyte Maturation

T_H lymphocytes are evidently important in nasopharyngeal colonisation. The binding of microbial antigen to a TLR initiates a complex cellular signal and a pro-inflammatory response is generated that will influence a T_H lymphocyte response [101]. The effector state that is adopted is influenced largely by the type of infection, PRR and APC [102, 103]. Nasopharyngeal tissue from children has been shown to express T_H1, T_H2, T_H17 and T_{reg} lymphocyte responses to allergen and antigen stimulation [75, 101, 104]. Additionally, the level of exposure of antigen is a strong influencing factor in what effector phenotype a T_H lymphocyte will adopt. Studies with bee venom have demonstrated that T_H lymphocytes with a T_H1 or T_H2 phenotype expressing IFN-γ or IL-4, respectively, switched to expression of the immunosuppressive cytokine IL-10

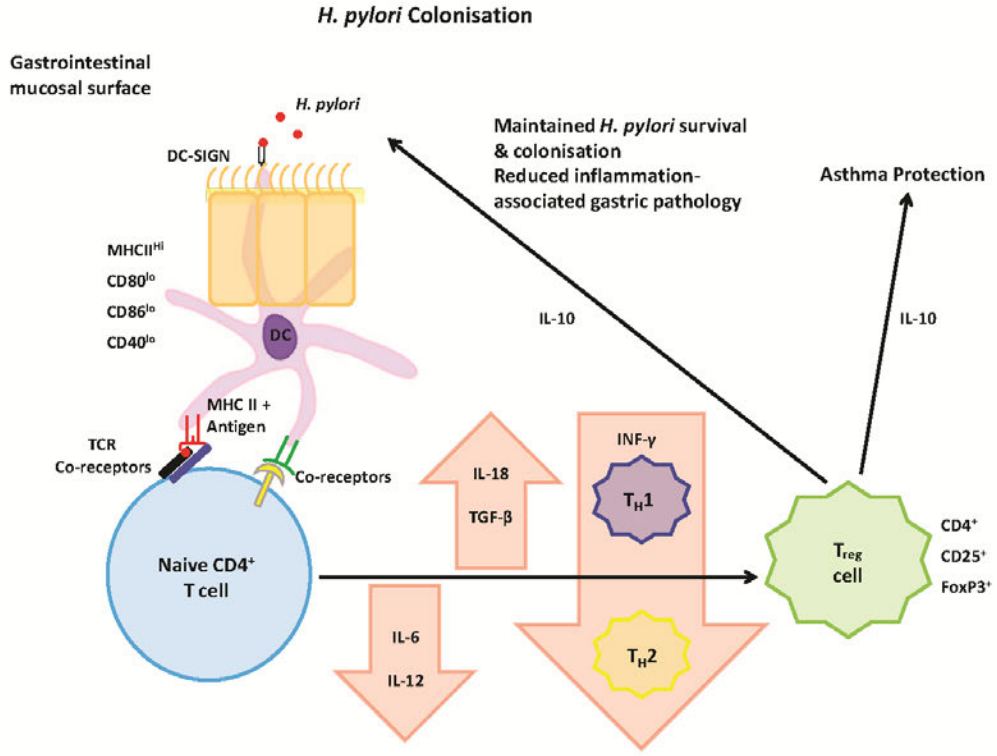

Fig. (3). The influence of *Helicobacter pylori* colonisation on dendritic cell maturation and T_H lymphocyte response in the gastrointestinal mucosa.

upon high dose, continual exposure to the bee venom allergen phospholipase A [105]. Evidently, the microbial or allergen environment that activates a DC and in turn primes a T_H lymphocyte will have a great impact on the outcome of the T_H lymphocyte phenotype and therefore on the effector response. It is known that microbial colonisation of the nasopharynx is high, but it remains to be determined if these elevated carriage loads influence the T_H lymphocyte phenotype and the nature of the effector response [104].

Linking Innate Immunity to T Helper Lymphocyte Responses in the Mucosa

In understanding immune regulation of nasopharyngeal colonisation, it is important to appreciate how the T_H lymphocyte response is influenced at a mucosal site abundant in microbial flora. An important component of the mucosa that detects microbes and signals to T_H lymphocytes are TLR of the innate immune system. The role of TLR in T_H lymphocyte activation has been elucidated in detail [76]. Experiments using MyD88-deficient knockout mice immunised with ovalbumin and complete Freund's adjuvant revealed that stimulated T lymphocytes failed to proliferate or to produce detectable levels of IFN-γ, a dominant cytokine of the T_H1 response. Furthermore, DC from these animals, when treated with mycobacteria, failed to exhibit up-regulated expression of any of the co-stimulators CD80, CD86, MHC class II and IL-12, all of which are important to formation of a T_H1 response. Taken together, these results demonstrate that the TLR signalling pathways in DC are influential in activating T_H lymphocytes and developing a T_H1 effector response [106]. It has also been shown that IL-6 from TLR-activated DC renders antigen-specific T lymphocytes to overcome the suppressive activity of T_{reg} lymphocytes, thereby skewing the immune response away from a T_H3 phenotype [107].

The concept of microbe- and PRR-driven activation of DC and T_H lymphocyte function in the mucosa is supported by studies with probiotics and *H. pylori* used to activate DC via the DC-specific intracellular adhesion molecule 3 (DC-SIGN). Such stimulation was shown to facilitate T_{reg} lymphocyte priming, a weakened T_H1 response marked by a decrease in IL-6 and an increased suppressive response via IL-10 (Fig. **4**) [108, 109]. In human gastric biopsy samples this phenomenon of *H. pylori*-induced T_{reg} lymphocyte

tolerogenic responsiveness by way of DC is corroborated by the demonstration that *H. pylori* binding to DC-SIGN causes a dampened T_H1 cytokine profile, characterised by reduced IFN-γ and IL-6 and an increased IL-10 immunosuppressive response. It is reasonable to speculate that the production of IL-10 may originate from a T_{reg} lymphocyte profile, although confirmation of this requires further research [110]. The mechanism of *H. pylori*-induced survival via DC to direct the T_H lymphocyte response to an immunosuppressive T_{reg} phenotype is clarified further by recent rodent model studies and supportive findings from human gastric biopsy samples [98]. Bone marrow-derived DC (BM-DC) and mesenteric lymph node (MLN)-DC co-cultured with *H. pylori* and *Escherichia coli* lipopolysaccharide (LPS) resulted in DC expressing high MHC class II and lower levels of CD80, CD86 and CD40 (MHC II^{HI} $CD80^{LO}$ $CD86^{LO}$ $CD40^{LO}$) compared to DC exposed to *E. coli* LPS alone, indicating *H. pylori* impaired DC maturation. Furthermore, IL-12 and IL-6 were decreased and IL-10 inversely elevated in the *H. pylori*-infected, *E. coli* LPS-stimulated cells compared to *E. coli* LPS stimulation only [98]. Interestingly, unlike reports that demonstrate DC-SIGN to be the ligand for *H. pylori* to DC, this study suggests that *H. pylori*-impaired DC maturation is independent of DC-SIGN. This conclusion should be treated with some circumspection since it is drawn from experiments conducted in mice transgenically expressing human DC-SIGN, in which *H. pylori* induced immature DC similar to those evident in wild-type mice, with no alternative receptors explored. Firstly, it is feasible that human DC-SIGN does not signal or function in mice as it does in humans and, secondly, if DC in mice express SIGNR3, the functional homologue of human DC-SIGN, it may be speculated that *H. pylori* preferentially binds SIGNR3 in mice as it does DC-SIGN in humans [109, 111]. Therefore, the above study conducted in $SIGNR3^{-/-}$ mice, and compared to $SIGNR3^{-/-}$ mice with transgenic expression of human DC-SIGN, will explore more accurately the possibility of *H. pylori*-impaired DC maturation independent of C-type lectin binding receptors. This research also demonstrated that *H. pylori*-experienced, semi-mature BM-DC and MLN-DC are adept at converting naive $CD4^+$ T_H lymphocytes to Fork-head box P3 $(FoxP3)^+CD25^+$ T_{reg} lymphocytes. Further, these tolerogenic DC are less able than non-*H. pylori*-experienced DC at activating an effector T lymphocyte response, indicated by reduced IFN-γ and T lymphocyte proliferative measures [98]. The ability of immature DC to generate $FoxP3^+CD25^+$ T_{reg} lymphocytes was found to be dependent on contact and transforming growth factor (TGF)-β, with DC-derived and T lymphocyte-derived IL-18 necessary to skew this response away from a T_H17, T_H1 profile to that of a T_{reg} lymphocyte. This was demonstrable in wild-type mice developing *H. pylori* tolerance while *Il18r*$^{-/-}$ mice had lower *H. pylori* colonisation levels together with higher gastric leukocytes, INF-γ and IL-17 production [98]. Together, these studies demonstrate how microbes and PRR are essential to directing adaptive cellular responses, often resulting in microbial tolerance at mucosal sites and prevention of inflammatory pathology.

The oropharynx and nasopharynx are other mucosal sites where T_H lymphocytes are induced via TLR-activated APC to induce host tolerance to commensal microbiota. Isolated oral Langerhans cells (LC) from human oral mucosa specimens are adept at inducing T_{reg} lymphocytes in the oral mucosa with immunosuppressive functionality. The process requires oral LC to mature via TLR4 activation, whereby up-regulation of co-stimulatory factors including CD80 and IL-10 occurs [112]. Co-culture of these active oral LC induces a T_{reg} lymphocyte phenotype producing IL-10 and TGF-β. Commensal oral bacteria are also reported to activate DC that in turn induce an immunosuppressive T_{reg} lymphocyte phenotype [112, 113]. The mechanism of T_{reg} lymphocyte activation in the human tonsil by DC has been recently elucidated (Fig. **4**). Immunohistochemical analysis of tonsil sections clearly shows the co-localisation of $FoxP3^+$ lymphocytes with $CD123^+$ plasmacytoid DC (pDC). Stimulation of pDC with TLR7 and TLR9 ligands up-regulates pDC expression of MHC II, CD80 and CD83 co-receptors [114]. Co-culture of these mature pDC with naive $CD4^+$ T lymphocytes, with or without TLR secondary stimulation, resulted in $CD4^+$ $CD25^+$ $FoxP3^+$ $CD127^-$ T_{reg} lymphocytes that secrete predominantly IL-10 and suppress proliferation of autologous T cells [114]. It is evident from these studies that TLR on APC such as DC are crucial to the crosstalk between innate and adaptive immunity that may influence the cellular response to microbes in the mucosa. These findings, taken together, provide strong evidence that T_{reg} lymphocytes may be activated at mucosal sites via DC sampling of the surrounding microenvironment. A comprehension of the induction of T_{reg} lymphocytes in the nasopharynx is crucially important for understanding the activation pathways and function(s) of T_{reg} lymphocytes in host tolerance to the milieu of microbial flora that colonises this site. Further investigation of T_{reg} lymphocyte induction mechanisms in different pathological states and in response to common commensals of the URT could provide novel approaches to coaching the immune system, through therapeutic interventions, towards faster recovery from infection and prevention of chronic illness.

T REGULATORY LYMPHOCYTES IN THE MUCOSA

T_{reg} lymphocytes are a subtype of $CD4^+$ T lymphocytes, the main function of which is the induction of peripheral immune tolerance to both foreign antigen and self antigen. A dysfunction or imbalance in T_{reg} lymphocyte numbers has been shown to contribute to the development of conditions such as allergy, cancer, autoimmune disorders and allograft rejection [115-118]. Chronic infections and inflammation-derived tissue damage may also arise from abnormal T_{reg} lymphocyte function [119, 120]. The cellular characteristics of T_{reg} lymphocytes are similar to those of a T_H lymphocyte, but they may be distinguished by their high level expression of FoxP3, a transcription factor belonging to the Fork-head – winged helix family. FoxP3 is necessary to maintaining the suppressive function of T_{reg} lymphocytes, its deletion resulting in the loss of suppressive capacity [121]. T_{reg} lymphocytes also express mid to high levels of the surface receptor CD25 (the IL-2 receptor α-chain), CD152, TNF receptor 2, membrane-bound TGF-β and particularly in humans, low level expression of CD127 (the IL-7 receptor α-chain). This low level CD127 expression is used, together with the other receptors mentioned, to distinguish T_{reg} lymphocytes from effector T lymphocytes. This is because FoxP3 expression cannot be used as a unique identifier of

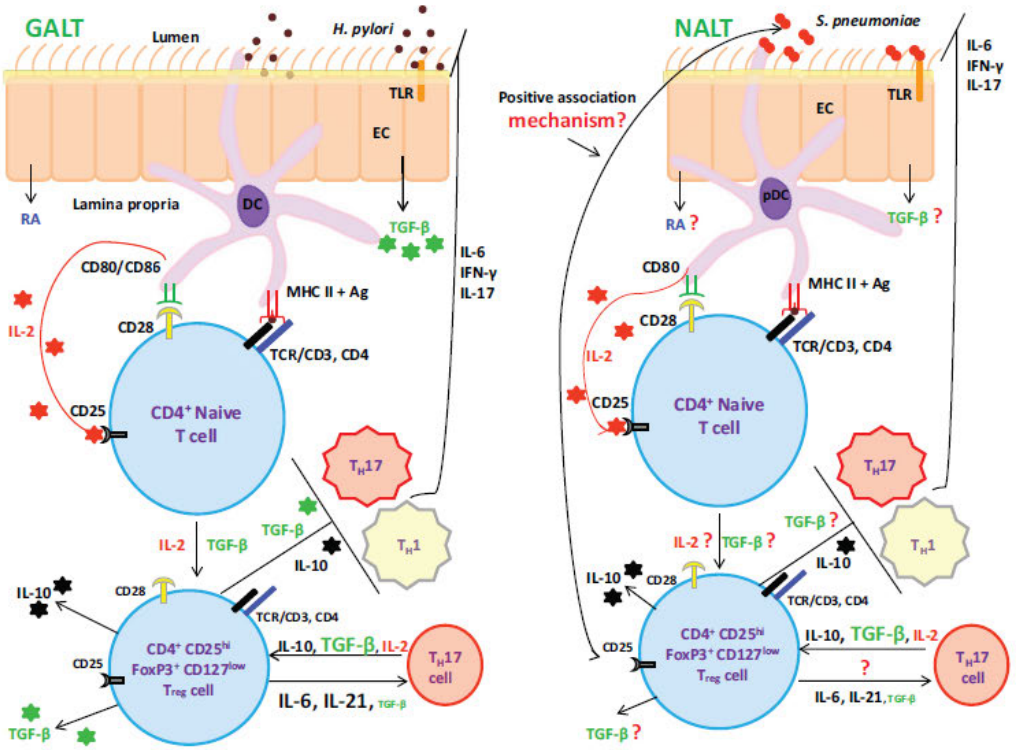

Fig. (4). T regulatory lymphocyte-induced host tolerance to commensal bacteria in the mucosa.

T_{reg} lymphocytes since effector T lymphocytes share FoxP3 expression [122, 123]. T_{reg} lymphocytes have been identified in the nasopharynx and are known to have a positive association with nasopharyngeal colonisation by *S. pneumoniae*. However, currently it is not known if host tolerance to *M. catarrhalis* and non-typeable *H. influenzae* colonisation is associated with a T_{reg} lymphocyte immunosuppressive response [104, 114]. Furthermore, the influence of polymicrobial colonisation of the nasopharynx on T_{reg} lymphocyte phenotype and effector response is yet to be elucidated.

T_{reg} Lymphocyte Subtypes

Two functional types of T_{reg} lymphocytes exist; naturally occurring, thymus-derived T_{reg} lymphocytes (nT_{reg}) and naive, $CD4^+$ $CD25^+$ inducible T_{reg} lymphocytes (iT_{reg}) [103]. iT_{reg} lymphocytes are generated from mature $CD4^+$ T lymphocytes, both conventional $CD4^+$ T and nT_{reg} lymphocytes, in the periphery at certain times of antigenic and cytokine stimulation. Both the cytokines TGF-β and IL-2 and retinoic acid (RA) are necessary for the transition of $CD4^+$ T lymphocytes to iT_{reg} lymphocytes. Type 1 iT_{reg} lymphocytes (Tr1) are known to secrete predominantly IL-10 during active immunosuppression, while Type 3 iT_{reg} lymphocytes (T_H3) exert immunosuppression via a biased secretion of TGF-β [124, 125]. All iT_{reg} lymphocytes may secrete these cytokines but the profile depends on the prevailing physiological setting [103, 124, 125]. The thymus-derived nT_{reg} lymphocytes possess a large TCR repertoire to self and non-self antigens in which their main role is to induce suppression of T lymphocytes and APC during autoimmune responses in a cell to cell contact-dependent fashion via CD152 and membrane-bound TGF-β [118]. They therefore have a strong influence in maintaining homeostasis. iT_{reg} lymphocytes on the other hand, have a more non-self-specific TCR repertoire which, when activated, exerts a suppressive effect on T lymphocytes and APC via soluble factors including IL-10 and TGF-β, rather than through a direct cell to cell contact mechanism [126]. As iT_{reg} lymphocytes are actively induced in the periphery via non-self antigen presentation and cytokine signals to suppress pro-inflammatory cellular responses, it is thought that they are involved in the tolerance to microbes at mucosal sites. Evidence of this has been discussed with regard to the gastrointestinal, oropharynx and nasopharynx mucosa where ligation of microbial receptors on DC induces a skewed T_{reg} immunosuppressive effector response, although precise mechanisms of this process in relation to

nasopharyngeal colonisation require clarification (Fig. **4**) [104, 109, 110, 112-114].

The Role of IL-10 and TGF-β in T_{reg} Lymphocyte Immunosuppression

The cytokines that T_{reg} lymphocytes generate to down-regulate pro-inflammatory responses by T_H and cytotoxic T lymphocytes, NK cells and APC exert immunosuppressive properties through a variety of mechanisms. IL-10 inhibits antigen presentation by blocking the co-receptors CD28 and CD80, thereby interrupting T lymphocyte stimulation, proliferation and cytokine production [127]. TGF-β and IL-10 are powerful immunosuppressors that can disrupt antigen presentation via changes in MHC Class I and II, CD40, CD80/CD86 and IL-12 co-stimulatory molecule expression on APC. These cytokines may inhibit the inflammatory process by disrupting effector macrophages and monocyte responses as well as affecting T_H1 and T_H2 responses by inhibiting potent pro-inflammatory cytokines and chemokines. IL-10 and TGF-β may also impair T lymphocyte activation via altering the CD28 signalling cascade [128, 129]. Overall, the pleiotropic immunosuppressive effects these cytokines exert under certain conditions of physiological stress inhibits antigen presentation and consequently T lymphocyte proliferation, activation and cytokine secretion. The actions of IL-10 and TGF-β driven by T_{reg} lymphocyte stimulation has been shown to be influenced by the physiological state (type of infection, allergy or autoimmune response), type of antigen and antigen exposure [105, 108, 128].

Maintenance of T_{reg} Lymphocyte Phenotype and Plasticity Characteristics

High expression of FoxP3, TGF-β and IL-2 each plays a role in maintaining iT_{reg} phenotype, inhibiting its conversion to a T_H17 lymphocyte phenotype under IL-6 stimulation [125]. Interestingly, the combination of these cytokines also has an impact on IL-6 signalling on nT_{reg} lymphocytes and can interrupt their switch to T_H17 lymphocytes [125, 130]. In light of this, T_{reg} lymphocytes may switch their function from an immunosuppressive role to an aggressive pro-inflammatory one depending on the cytokine environment and transcription factor activation. Under the influence of low levels of TGF-β and high levels of IL-6, IL-21 and IL-23, driven by the transcription factor retinoic acid-related orphan receptor γt, nT_{reg} but not iT_{reg} lymphocytes may switch to an IL-17-producing T_H17 pro-inflammatory response (Fig. **4**). The ability of these cells to switch their role from passive, anti-inflammatory mediators to aggressive, pro-inflammatory inducers, under the influence of a fine balance of regulating factors, makes them key players in maintaining optimal health at mucosal sites where constant microbial stimulation is endured [130].

T_{reg} Lymphocytes and Microbial Interactions

Several studies performed in gastrointestinal tissue have outlined the relationship among colonising bacteria and T_{reg} lymphocytes. Positive correlations are evident with *H. pylori* colonisation, infection and related inflammation [10, 78]. In the state of inflammation it may be reasoned that T_{reg} lymphocytes are present to control the inflammatory response, yet asymptomatic *H. pylori* colonisation associates positively with the presence of T_{reg} lymphocytes [10, 78]. It is thought that this positive correlation is driven by the ability of *H. pylori* to induce naive $CD4^+$ T lymphocytes via DC, TGF-β and IL-10 to mature into $CD4^+$ $CD25^+$ $FoxP3^+$, IL-10-producing iT_{reg} lymphocytes and away from a T_H17 phenotype. This results in prevention of a targeted T_H17 response to *H. pylori* colonisation via a skewed iT_{reg} phenotype (Fig. **4**) [110]. Intestinal colonisation in mice with altered Schaedler flora has also contributed to knowledge of the role of T_{reg} lymphocytes in host-microbe homeostasis. These mice show increased iT_{reg} lymphocytes with established colonisation and consequently T_H17 and T_H1 responses are down-regulated, thereby preventing inflammation and promoting bacterial colonisation in the intestine [77]. Interestingly, the influence of probiotics on improved gastrointestinal health, in particular the anti-inflammatory benefits, may be through induction of IL-10-producing T_{reg} lymphocytes via stimulation of DC and naive $CD4^+$ T lymphocytes by probiotics such as *Lactobacillus casai*, *L. reuteri* and *Bifidobacterium infantis* [108, 131].

T_{reg} lymphocytes have been isolated from the oral cavity of patients with periodontitis and gingivitis lesions, although their association with bacterial colonisation in this site is still poorly understood. Recent evidence indicates T_{reg} lymphocytes with tolerogenic functions are induced in the oral mucosa via oral LC or DC activated with TLR4 or *Streptococcus mitis*, *Propionibacterium acnes* and *Bacteroides fragilis*, respectively [112, 113, 132]. Although this suggests a link between oral commensals and T_{reg} lymphocytes, further research needs to be undertaken to confirm the T_{reg} lymphocyte phenotype since FoxP3 data were lacking in these studies.

Mycobacterium tuberculosis has been demonstrated to induce T_{reg} lymphocytes with suppressive capacity *in vitro* through monocyte activation and prostaglandin E2 production [133]. Of note, T_{reg} lymphocytes were elevated in peripheral blood mononuclear cells (PBMC) of tuberculosis patients compared to PBMC from healthy tuberculin reactors, indicating a direct relationship between the suppressive T lymphocyte subset and susceptibility to primary tuberculosis [133]. The persistence of malaria and human papillomavirus (HPV) infections has also been linked with elevated levels of functional T_{reg} lymphocytes. In patients with a clinical *Plasmodium falciparum* infection, blood parasitaemia increased simultaneously with TGF-β peaks in serum, $CD4^+$ $CD25^{hi+}$ T lymphocyte increases and raised expression of FoxP3 in PBMC. IL-6 and IFN-γ were measured at lower concentrations and a slower release, indicating that a T_H1 response was under the suppressive effects of a T_{reg} lymphocyte response and consequently favouring a persistent *P. falciparum* infection. Persistent HPV16 infection was also shown to have a positive association with an increased percentage of circulating T_{reg} lymphocytes [134, 135]. However, not all persistent infections are a result of T_{reg} lymphocyte-mediated tolerance. *P. aeruginosa* induces T_{reg} lymphocytes in the spleen and lungs of infected mice, but no relationship is evident between T_{reg} lymphocytes and *P. aeruginosa* infection [136]. This may be due to the high levels of IL-6 in this type of aggressive infection, thereby overcoming a T_{reg} lymphocyte

response. This highlights the different roles of T_{reg} lymphocytes in colonisation and infection [136].

Several insightful studies have been published recently which focus on T_{reg} lymphocyte responses to commensals of the nasopharynx. In human tonsils, *Neisseria meningitidis*-specific T_{reg} lymphocytes have been identified that display regulatory activity towards suppression of the T_H1 dominant response to *N. meningitidis* [137]. Even more surprising was that this activity was observed only in the tonsil mononuclear cells and not in PBMC, indicative of a compartmentalised mucosal response [137]. Similar findings have also been reported with adenoid-derived T_{reg} lymphocytes responsive to *S. pneumoniae* colonisation of the nasopharynx, but in this example a positive correlation was also evident between the suppressive activity and frequency of T_{reg} lymphocytes and the increased carriage of *S. pneumoniae* [104]. Both of these studies on nasopharyngeal commensals highlight the ability of upper respiratory mucosal-derived T_{reg} lymphocytes to induce host tolerance and hence to promote survival of commensals in the nasopharynx. Furthermore, T_{reg} lymphocyte-mediated immune suppression to nasopharyngeal commensals is compartmentalised to the mucosa, leaving one to speculate that T lymphocyte responses to bacterial colonisers (and in events of physiological stress, potential pathogens) of the upper airways may be regulated at the site of induction as opposed to systemically controlled responses [104, 137].

CONCLUSION

While the first steps to dissecting tolerance to commensals in the upper respiratory tract have been taken, there are still many cellular mechanisms involved in this process that are yet to be characterised (Fig. **4**). Investigation of the adenoid and peripheral blood T_{reg} lymphocyte responses to nasopharyngeal colonisers, in conjunction with clinical nasopharyngeal culture outcomes, would be beneficial to understanding colonisation tolerance in the respiratory mucosa. This would increase our knowledge of host tolerance to nasopharyngeal colonisation in OM-prone children. From a clinical research perspective, this may reveal novel strategies for immune therapy to regulate nasopharyngeal colonisation, with the ultimate goal of preventing progression to chronic disease.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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The full article citation is listed in the section ‘Publications Arising’ in the preamble of this thesis.

2.2 Supplementary Information

The publication "*The balancing act between colonisers and inflammation: T regulatory and T_H17 cells in mucosal immunity during otitis media*", provides much information regarding the respiratory system, OM and associated immunity, and immune regulation; all concepts relevant to this study. Additional topics will be covered herein, to provide the necessary background information relevant for this study.

2.3 Adenoidectomy and Tonsillectomy

Often children will develop COM whereby the child becomes unresponsive to antibiotic therapy and another course of action needs to be implemented in order to help improve the child's health status. It is in severe cases such as these that clinicians will encourage adenoidectomy and tonsillectomy to remove inflamed and damaged tissue (Hakim 2003). Consequently, an overwhelming population of OM pathogens that have internalised in the tissue or grown as impenetrable biofilms on the surface of the lymph tissue are also removed which appears to render the child less likely to develop bacterial OM post-surgery (Heiniger et al. 2007; Hoa et al. 2009). Adenoidectomy and tonsillectomy may also be performed for chronic tonsillitis or hypertrophied adenoids and tonsils causing orofacial growth abnormalities, sleep apnea, dysphagia and airway obstruction (Hakim 2003).

2.4 Complications and Sequelae of OM

Depending on the severity of OM, complications and sequelae may develop. Perforation of the tympanic membrane (TM) may occur due to the accumulated pressure within the middle ear and the weakness of the TM due to inflammation. When perforation occurs, otorrhea usually ensues. The perforation may heal as the infection and OM resolves, however if the perforation is severe, often called a ruptured ear drum, it may not heal optimally and a chronic perforation may develop. Once the perforation heals, however, the associated scarring frequently results in hearing loss (Bluestone 2000). A perforated TM or a retracted TM (atelectasis of the middle ear) may cause the development of a cyst within the middle ear or the mastoid and this is known as a cholesteatoma and which may need to be removed surgically to avoid further hearing loss. A further additional complication of OM is inflammation of the mastoid, or mastoiditis, and this may present as acute, subacute or chronic inflammation, and the scarring and tissue remodelling in this condition may also lead to hearing loss (Bluestone 2000).

2.5 Epidemiology of OM

The prevalence of OM varies among different ethnic populations (Klein 2001). The nature of this phenomenon remains unclear, although genetic predisposition, socio-economic status, quality of education, child rearing in large extended families, living in a tobacco-smoking environment and community health care availability are all contributing factors to developing OM (Morris et al. 2005). Australian Aborigines as well as Native Americans, Alaskans and Canadians are ethnic populations at high risk for developing OM (Klein 2001; Morris et al. 2005). In a large scale survey spanning several Aboriginal communities throughout Central and Northern Australia, with over 700 children aged 6 to 30 months enrolled in the study, an astounding 90 percent of the children examined showed evidence of an OM event (Morris et al. 2005). Only 20 percent of these Aboriginal children are expected to have normal hearing as they present extraordinarily high incidences of severe OM (with perforation). By 2 years of age 45 percent of children in the study had a documented perforation, in comparison to other regions which rarely document rates higher than 5 percent. Unfortunately, children as young as 19 days of age have documented perforations, indicating that infections are contracted early in life, resulting in the child experiencing developmental and learning difficulties due to hearing impairment (Morris et al. 2005).

2.6 Colonisation in the Upper Respiratory Tract

M. catarrhalis and NTHi are both aerobic Gram negative bacteria that colonise the human oropharynx and nasopharynx mucosa. *M. catarrhalis* is a diplococcus bacterium while NTHi is a cocco-bacillus bacterium (St. Geme III 2000; Forsgren et al. 2001). *S. pneumoniae* is a Gram positive, facultative anaerobic, encapsulated diplococcus bacterium (Robinson et al. 2001; Glover et al. 2008).

Investigating upper respiratory tract (URT) colonisation is important in order to gain an understanding of OM. The nasopharynx is a natural reservoir for bacterial commensals that may shift to OM pathogens (Faden et al. 1997). Biotyping and deoxyribonucleic acid (DNA) fingerprinting identifies that bacterial strains recovered from the middle ear during an OM infection are the same strains of bacteria found in the nasopharynx. This indicates that migration of these bacteria from the nasopharynx to the middle ear may occur via Eustachian tube dysfunction and/or predisposed viral infection (Loos et al. 1989). The nasopharyngeal carriage rate of NTHi is related to the incidence of OM. One previous study

showed that NTHi strains found in the nasopharynx are found simultaneously in the middle ear fluid from OM prone children and that this phenomenon occurred in 66 percent of cases (Dhooge et al. 2000).

The predominance of colonising strains often shift with the elimination of some strains and replacement with different ones. In one study examining NTHi trends in colonisation of remote Aboriginal infants, several children could carry up to 10 different ribotypes of NTHi over a 9 month period (Smith-Vaughan et al. 1997). In addition, children were often culture positive for multiple ribotypes simultaneously and some ribotypes were culture positive in different infants simultaneously. Similarly, an unrelated study shows dissemination of NTHi strains between siblings (Dhooge et al. 2000). Therefore, children who live in over-crowded households are at a higher risk of acquiring high nasopharyngeal carriage rates of NTHi, possibly of multiple strains and therefore are at an increased risk of developing OM (Smith-Vaughan et al. 1997). Another example of colonisation shift is with the introduction of the 13-valent pneumococcal conjugate vaccine (PCV-13), which protects against 13 serotypes of *S. pneumoniae*. With the implementation of PCV-13 in the last 5 years, *M. catarrhalis* has been reported as the most prevalent pathogen in OM in regions of the United States (Casey et al. 2015). It seems that with the reduction of pneumococcal carriage due to PCV-13, microbial competition has lessened, enabling *M. catarrhalis* to enhance its colonisation of the nasopharynx. The immune factors that regulate host tolerance to such colonisation shift at an early age are not well understood, and require greater priority in ongoing research (Jacoby et al. 2007).

NTHi may also internalise into the macrophage-like cells of the adenoids and form biofilm structures on the surface of the adenoids (Dhooge et al. 2000; Hoa et al. 2009). By using histological techniques with fluorescent *in situ* hybridization (FISH), *M. catarrhalis* has also been detected on adenoid and tonsil tissue, where it resides beneath the epithelium, invading the surrounding tissue and co-localising with macrophages and B lymphocytes (Heiniger et al. 2007). Remaining beneath the epithelium in these tissues provides a safe reservoir for both NTHi and *M. catarrhalis* as the bacteria may remain undetected by pharyngeal surface sampling at this site. Unfortunately, this means that the nasopharyngeal lymphoid tissue may possibly be a source of endogenous re-infection (Heiniger et al. 2007; Hoa et al. 2009).

The rate of bacterial colonisation, composition and bacterial load has been associated with the onset of OM (Watson et al. 2006). *M. catarrhalis* is the earliest coloniser of the nasopharynx, followed by *S. pneumoniae* and NTHi, with all three bacteria documented to colonise the nasopharynx of infants less than 1 month of age (Faden et al. 1997; Watson et al. 2006). In a study examining bacterial colonisation among Aboriginal and non-Aboriginal children, by 2 years of age 96 percent of the Aboriginal population cultured positive for colonisation of at least one of the three pathogens for OM. Additionally, multiple causative bacteria were cultured concurrently from the children, however, this trend was more pronounced in Aboriginal children (Watson et al. 2006). The study also highlighted the increased colonisation rates for *M. catarrhalis*, *S. pneumoniae* and NTHi in Aboriginal children at 50, 49 and 41 percent, compared to colonisation in non-Aboriginal children at 25, 25 and 11 percent, respectively. It is understood that the increased colonisation loads of these bacteria raise the risk of developing OM, but what is unclear is why this population has such high carriage rates. Moreover, there is a lack of understanding of the mechanisms within the immune system that are responsible for induction of tolerance to such high bacterial loads (Watson et al. 2006). These differences are central to explaining the high prevalence of OM in Aboriginal children as early colonisation and frequency of colonisation correlates positively with early episodes and frequency of AOM (Leach et al. 1994; Faden et al. 1997).

2.6.1 Bacterial Interactions

The way bacteria network will influence nasopharyngeal bacterial colonisation and infection. In children, *S. pneumoniae* colonisation may alter depending on interactions with other microbial species in the ecological niche of the nasopharynx. A study in Aboriginal and non-Aboriginal children showed positive correlations for *S. pneumoniae* colonisation in the presence of Rhinovirus (RV) or *M. catarrhalis* (Jacoby et al. 2007). The positive relationship between these bacteria in the presence of respiratory virus has also been documented in several other polymicrobial studies, demonstrating increased and prolonged carriage loads of *S. pneumoniae* (Krishnamurthy et al. 2009; Dahlblom & Söderström 2012). Co-colonisation of *S. pneumoniae* with *Neisseria meningitidis* is also reported to have a positive association (Pericone et al. 2000; Dahlblom & Söderström 2012). When different serotypes of *S. pneumoniae* are co-cultured, one serotype can also exert inhibitory effects on a neighbouring serotype. While the polymicrobial environment is not fully

understood, these observations may be explained simply by the competitive nature of the bacteria (Lipsitch et al. 2000). Interestingly, negative associations are evident with *S. pneumoniae* and *H. influenzae*; however, when these two nasopharyngeal commensals are together with *M. catarrhalis* in a triple co-colonisation setting, or when co-infected with influenza B virus, *S. pneumoniae* recovery is enhanced (Brunstein et al. 2008; Pettigrew et al. 2008; Krishnamurthy et al. 2009; Dahlblom & Söderström 2012). This phenomenon is reflected in a clinical setting where the risk of developing OM in Australian Aboriginal infants increases by 30-fold when early colonisation is established with *M. catarrhalis* and *S. pneumoniae* or NTHi, compared to *M. catarrhalis* single colonisation of the nasopharynx (Leach et al. 1994). *S. pneumoniae* also showed an increase in incidence of infections when Respiratory Syncitial Virus (RSV)-B was present (Brunstein et al. 2008). This may be explained by *S. pneumoniae* binding the RSV-B glycoprotein present on the surface of RSV-B-infected cells to enhance its ability to adhere to host cells and cause infection (Hament et al. 2005). Both positive and negative correlations between *S. pneumoniae* colonisation in the presence of adenovirus or *S. aureus* have been reported, but this was age-dependent with the positive affect occurring between 10 to 14 months during peaks in pneumococcal colonisation (Jacoby et al. 2007). Collectively, these studies demonstrate the complexity of nasopharyngeal colonisation, showing that the inclusion of respiratory virus and neighbouring bacteria may affect how bacteria co-colonise and influence the progression of OM infections.

2.7 Nasopharyngeal Commensals and Infection

The three bacteria discussed demonstrate similar colonisation trends, but each is quite distinct in its ability to cause infection. Arguably, *S. pneumoniae* is the most virulent bacterium of the airways in children, as over one million childhood deaths occur each year as a direct result of *S. pneumoniae* infections (O'Brien & Nohynek 2003). In 1998 there were in excess of 62 000 cases of invasive pneumococcal disease reported in the United States alone and of these more than 6 000 had a fatal outcome (Robinson et al. 2001). Systemic diseases caused by invasive *S. pneumoniae* include bacteraemia, meningitis, pneumonia, arthritis and osteomyelitis, and peritonitis (Taylor & Sanders 1999; Robinson et al. 2001; Martens et al. 2004). Similarly to *S. pneumoniae*, *M. catarrhalis* has been known to cause childhood pneumonia, acute bronchitis, laryngitis, keratitis and one fatal case of meningitis, however these are documented in case reports and are quite atypical for

the diseases caused by *M. catarrhalis* (Vaneechoutte et al. 1990a; Heiskanen-Kosma et al. 1998; Jin 2000). *M. catarrhalis* is associated more commonly with the exacerbation of chronic obstructive pulmonary disease (COPD) and chronic bronchitis in adults, and causes pneumonia in the elderly, emerging as a nosocomial respiratory pathogen (Vaneechoutte et al. 1990b; Verghese et al. 1990; Murphy et al. 2005a; Al-Anazi et al. 2007). Systemic cases of invasive *M. catarrhalis* infection are observed in cultures from blood and pleural fluid which are usually recovered from patients with lower respiratory tract infections in which the organism has played an aetiological role (Sugiyama et al. 2000). NTHi is also associated with the pathogenesis and exacerbations of cystic fibrosis (CF) and COPD. Evidence of NTHi bacterial cultures, biofilm formations and internalisation in the lung tissue enables this bacterium to cause pneumonia and bronchitis, thereby making it a commonly occurring pathogen of the lower airways (Starner et al. 2006a; Erwin & Smith 2007).

In order to combat the challenge of invasive disease, the 7-valent pneumococcal conjugate vaccine (PCV-7) was introduced in late 2000, since when it has been shown to be of some benefit as documented incidences of childhood invasive pneumococcal disease caused by serotypes of the vaccine have declined. Unfortunately, the replacement of vaccine serotypes with non-vaccine serotypes has been a major problem and as a consequence the disease burden remains high (Aguiar et al. 2008). Approximately 20 of the 90 serotypes are responsible for the majority of pneumococcal disease, with invasive disease associated with serotypes 1, 3, 4, 6B, 7F, 14, 18C and 23F (Brueggemann et al. 2003; Martens et al. 2004; Hammerschmidt et al. 2005). PCV-7 has been shown to be highly effective in reducing invasive pneumococcal disease in both children and adults, as well as reducing the incidence of OM by 5 to 10 percent (Pittet & Posfay-Barbe 2012). Since the license for public administration of PCV-7 was granted, a similar 13-valent conjugate vaccine and an un-conjugated 23-valent polysaccharide vaccine have been introduced, but to date there is little evidence of their impact against pneumococcal disease as both vaccines were introduced only recently (Pittet & Posfay-Barbe 2012). Furthermore, in a recent study to observe nasopharyngeal and middle ear isolation of OM pathogens in children 2 to 4 years post PCV-7 regime, NTHi was highlighted as the most prominent pathogen in children with a history of recurrent AOM (rAOM) (Wiertsema et al. 2011). This may be associated with reports that the PCV-7 immunisation regime has influenced the increase of NTHi and *S. aureus* nasopharyngeal colonisation, which may impact on local and systemic infections

associated with these pathogens (Spijkerman et al. 2012). Due to such unwanted side-effects, there is a necessity for improved immunological protection against NTHi. In light of this, clinical trials have shown that a novel pneumococcal NTHi protein D conjugate vaccine (PHiD-CV) decreased carriage rates and AOM caused by NTHi by nearly 40 percent, yet the impact on NTHi colonisation has been variable and inconsistent in literature (Wiertsema et al. 2011; van den Bergh et al. 2013). These trials demonstrated some clinical significance for the PHiD-CV and the therapeutic potential that this vaccine candidate may have for developing a stronger acquired immunological defence against NTHi.

NTHi, *M. catarrhalis* and *S. pneumoniae* cause local infections including OM and sinusitis, with *M. catarrhalis* accounting for 5 percent and 20 percent of cases respectively, while nearly 40 percent of OM cases are caused by the primary pathogen *S. pneumoniae* (Commisso et al. 2000; Broides et al. 2009). In the United Kingdom more than 630 000 general practitioner visits occur annually due to pneumococcal OM (Farrell et al. 2008). NTHi is the second most dominant causative pathogen of OM, accounting for approximately 20 to 30 percent of AOM, 40 percent of COME and it is the leading pathogen for rAOM (St. Geme III 2000; Webster et al. 2006). The only current prevention and treatment options against these pathogens in OM are the above-mentioned vaccines which locally only provides protection against vaccine type pneumococcal disease and not OM caused by other otopathogens, or antibiotic therapy for which the risk lies in over-prescribed use leading to emergence of antibiotic-resistant pathogens (Daly et al. 2010). Therefore, further investigations into the human body's natural defence against these pathogens and vulnerabilities associated with them may facilitate treatment options against OM pathogens that provide an alternative to the current therapies that are limited in their efficacy.

2.7.1 Microbial Virulence Factors Involved in the Induction of Infection

OM pathogens have unique characteristics that enable them to adhere to the respiratory mucosal surface, internalise into the cells and initiate infection. Colonisation and infection by the bacteria is based primarily on their adept cellular adherence and evasion of the host immune response. There are various surface antigens and virulence factors described for the bacteria in Tables 2.1, 2.2 and 2.3 that play a crucial role in this process (Pracht et al. 2005; Webster et al. 2006; Slevogt et al. 2008). Phosphorylcholine (ChoP), a constituent of the cell wall of pneumococcus, shows molecular mimicry with human carbohydrate

structures that bind the platelet-activating factor (PAF) receptor (PAFr). Interestingly, ChoP has been demonstrated to be a pneumococcal and NTHi ligand for the PAFr and to facilitate adherence and internalisation of both of these bacteria into host epithelial cells (EC) (Cundell et al. 1995; Swords et al. 2001). The PAFr is utilised as a docking point on cells only by viable pneumococci and after an inflammatory response, suggesting that it is an active response by these bacteria to shift from colonising commensal to invading pathogen (Cundell et al. 1995). It is believed that respiratory viruses play a role in this process as they provoke the production of pro-inflammatory cytokines in respiratory cells during infection, as a consequence of which the surface expression of the PAFr is up-regulated and pneumococcal and NTHi adherence, colonisation and subsequent entry into the cells are enhanced (Ishizuka et al. 2003). Pneumococcus and NTHi also share the ability to bind the RSV glycoprotein expressed on RSV-infected EC. This is yet another example of how respiratory viral infections enhance bacterial infections (Avadhanula et al. 2007). NTHi has also been shown to bind the intercellular adhesion molecule 1 (ICAM1) and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) receptors on host EC via P5 fimbriae on its cell wall as an initial adherence mechanism prior to internalisation and infection (Avadhanula et al. 2006). Unlike either *S. pneumoniae* and NTHi, *M. catarrhalis* does not express ChoP, but does express the ubiquitous surface proteins (Usp) known as UspA1 and UspA2 that dock to the CEACAM1 receptors on respiratory EC to facilitate its adherence and internalisation of host cells (Slevogt et al. 2008).

Table 2.1 Pneumococcal proteins & their function in the host.

Pneumococcal Virulence Factors	**Function**
PspA	Binds to N-acetyl-glucosamine Prevents C3 binding to pneumococcal surface Binds to lactoferrin
CbpA	Binds to D3 and D4 domains of pIgR – facilitates migration through mucosal barrier Affinity for sialic acid and lacto-N-neotreatose on host cell surface
ChoP	Binds to PAFr on host cells Molecular mimicry of human PAF – possible evasion of host immune response
Ply	Cytolytic toxin – activates complement Forms pores in host cells
NanA, BgaA, StrH	Cleave N-acetylneuraminic acid associated with mucin Cleave glycoproteins, glycolipids and oligosaccharides; may facilitate increased exposure of receptors on EC Promote resistance to opsonphagocytic killing
Hyl	Reduces hyaluronan components of extracellular matrix
PavA	Binds to fibronectin

BgaA, β-galactosidase, CbpA, choline-binding protein A, ChoP, Phosphorylcholine, Hyl, hyaluronate lyase, NanA, Neuraminidase, PAF, platelet-activating factor, PAFr platelet-activating factor receptor, PavA, pneumococcal adhesion and virulence A, pIgR, polymeric Ig receptor, Ply, Pneumolysin, PspA, pneumococcal surface protein A, StrH, β-N-acetylglucosaminidase (Cundell et al. 1995; Magee & Yother 2001; Pracht et al. 2005; Rajam et al. 2007; Dalia et al. 2010).

Table 2.2 M. catarrhalis proteins & their function in the host.

***M. catarrhalis* Proteins**	**Function**
UspA1/UspA2	Binds to N-domain CEACAM1 Adherence to host cells Adheres to fibronectin Involved with serum resistance
OMP CD	Adherence to host cells
OMP E	Porin
TFP	Nasopharyngeal colonisation in an *in vitro* model of Chang EC
LOS	Molecular mimicry of the human antigen Pk. May be attributable to colonisation of EC as a redundancy in evasion of the host's immune response
MID/Hag	Adherence to host cells
McaP	Autotransporter/phospholipase B Adherence to host cells

UspA1/UspA2, ubiquitous surface protein A1 and A2, CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1, LOS, lipooligosaccharide, McaP, *M. catarrhalis* adhesin protein, MID/Hag, *M. catarrhalis* IgD-binding protein, OMP CD, outer membrane protein CD, OMP E, outer membrane protein E, TFP, type IV pili (Holm et al. 2003; Bullard et al. 2005; Lipski et al. 2007; Luke et al. 2007; Slevogt et al. 2008).

Table 2.3 NTHi proteins & their function in the host.

NTHi Proteins	Function
OMP Hap	Binds to fibronectin, laminin and collagen IV
OMP Hia	Receptor unknown
OMP Hsf	Binds to vitronectin
IgA1 Protease	Cleaves IgA1
	Autotransporter family proteins
Protein 6	Binds to TLR2
	Involved in bacterial resistance to serum complement
HMW 1 and HMW 2	Mediate attachment to EC
Protein 2	Binds to mucin proteins
P5 Fimbriae	Binds to CEACAM1 and ICAM1
OMP D	Binds IgD
OMP E	Recognised by IgDλ myeloma
	Involved in adherence and internalisation into host cells
LOS	Binds TLR4
	Involved in resistance to host killing
ChoP	Binds to PAFr on host epithelia
	Involved with evasion of host inflammatory response and the formation of persistent biofilms

OMP, outer membrane protein; IgD, immunoglobulin D; TLR2, toll-like receptor 2; HMW, high molecular weight; CEACAM1, carcinoembryonic antigen- related cell adhesion molecule 1; LOS, lipooligosaccharide; ChoP, phosphorylcholine; ICAM1, intercellular adhesion molecule 1; PAFr, platelet-activating factor receptor; (Webster et al. 2006; Hong et al. 2007; Ronander et al. 2008).

Although the bacteria have the means to adhere to and internalise within host cells, they also demonstrate other qualities that can aid their escape from host immune responses. Pneumococcus possess a polysaccharide capsule that envelopes the bacteria to provide protection from phagocytosis and which also provides the basis for serotyping (Dalia et al. 2010). Unlike pneumococcus, NTHi lacks an outer polysaccharide capsule, so therefore it cannot be serotyped and remains difficult to develop effective vaccine therapies against it (Erwin & Smith 2007). Despite pneumococcus having a protective polysaccharide capsule, like NTHi and *M. catarrhalis* the bacteria remain vulnerable to severe innate and adaptive immune responses. Therefore, they internalise into host cells of the airways in an attempt to gain protection from the host's immune defences (Erwin & Smith 2007). OM pathogens also share a common factor of the production of different proteases and toxins involved in

attacking components involved with chemical and cellular defences. NTHi produces IgA1 proteases that cleave mucosal IgA1 of the innate response, while *S. pneumoniae* produces pneumolysin (Ply), a potent toxin that lyses host cells by creating pores in the cell wall, thereby enabling evasion of adaptive cellular responses (Rubins & Janoff 1998; St. Geme III 2000). More than 90 percent of *M. catarrhalis* strains produce β-lactamase, an enzyme that breaks down the β-lactam family of antibiotics, rendering *M. catarrhalis* and neighbouring bacteria resistant to most β-lactam antibiotic therapies (Hoban et al. 2001).

One of the most notably impressive mechanisms that all the OM pathogens employ is the production of various carbohydrates and proteins which form a bacterial matrix or biofilm structure. In the state of a biofilm, the bacteria remain impenetrable to cellular and chemical defences of the host immune response as well as to antibiotic therapy. As a result, the bacteria central to the biofilm remain unharmed and intact pathogens of the nasopharynx and middle ear (Hoa et al. 2009). Evidence of biofilm structures has been identified in cases of CF and COPD (Murphy et al. 2005b; Starner et al. 2006b). Tympanostomy tubes removed from children with OM also show evidence of biofilm formations. Although the species were not determined, it is plausible that any of the OM pathogens discussed may have been the causative bacterium due to their known ability to form biofilms *in vivo* in clinical and experimental settings, as well as being dominant pathogens in OM (Post 2001; Hoa et al. 2009). Hence, it seems that these virulent pathogens are almost indestructible. Fortunately, the bacteria are not at all invisible and the antigens that are designed to protect them will often activate an immune reaction by the host involving complement, cytokine cascades and inflammatory responses. An aggressive host response may clear an infection, although this may be at the cost of damage to host cells from both bacterial toxins and severe inflammatory responses (Magee & Yother 2001). In order to avoid such consequences, the immune system employs mechanisms to regulate the inflammatory response in times of prolonged stress, and thus at colonised mucosal sites there is a fine balance between immune homeostasis and microbial overgrowth (Lan et al. 2007).

3 RESEARCH DESIGN AND DEMOGRAPHICS

3.1 Experimental Design

This study investigated cellular immunity in 20 COM prone and 20 non-COM prone children between 2 and 7 years of age. The aims were to investigate the proportion of immune cell populations relative to total lymphocytes in the adenoids and blood from consenting participants, and to identify correlations, if any, between the cell populations, clinical parameters, and demographic factors. In order to achieve this, adenoid and blood samples collected from participants were subject to *ex vivo* cell culture and flow cytometry techniques to measure cellular markers to determine cell types and their relative percentages. Nasopharyngeal aspirates and adenoid biopsies were cultured to determine bacterial carriage. Using enzyme-linked immunosorbent assays (ELISA), *S. pneumoniae*, whole cell sonicate antigen (WCSA)-specific total IgG and total IgA titres were measured in plasma and/or saliva samples respectively, to determine in further detail the immune cellular responses to *S. pneumoniae* colonisation in children. Demographic and clinical information was collected from participant questionnaires and medical records (see Appendix A and B). Finally data from the lymphocyte analysis, clinical microbiology, ELISAs, participant questionnaires and clinical records from the participants were collated and subjected to statistical analysis to determine the significance of correlations between experimental data and clinical outcomes and to identify distinct trends between COM prone and non-COM prone populations. OM risk factors were not expected to be different from those identified elsewhere, although it was necessary to identify them here in order to determine their association with COM proneness in this study cohort. OM risk factors were also assessed to meet primary outcomes where risk factors were correlated with lymphocyte populations to determine relationships present, if any. Table 3.1 details the demographic, environmental, clinical and microbiological factors included in the study, while Table 3.2 outlines the experimental groups of the study.

Table 3.1 Biological, demographic, clinical & microbiological factors

Biological	**Demographic**	**Clinical**	**Clinical Microbiology**
Whole adenoid via curette method	Age	COM diagnosis	*S. pneumoniae*
NPA, approximately 10 mL via the saline syringe nasal aspirate method	Sex	Respiratory tract infection diagnosis (including tonsillitis and/or OM) (URTI)	*M. catarrhalis*
Blood, 10 mL via venipuncture	Aboriginal and Torres Strait Islander Heritage	Airway obstruction / AH	NTHi
	Number of children in household ≤15 years of age	Clinical reason for adenoidectomy	*S. aureus*
	Child's birth order – youngest, middle or eldest child among siblings	Antibiotic history	*Streptococcus pyogenes*
	Siblings with a history of OM	Steroid history	Group A Streptococcus
	ETS exposure	Grommets history	*Alloiococcus otitidis*
	Childcare/kindergarten/preschool/school attendance (childcare)	Pneumococcal conjugate vaccine history	*Pseudomonas aeruginosa*
	Routine immunisation compliance	Clinical microbiology from adenoid biopsy and NPA	Other organism (noted)

AH = adenoid hypertrophy; COM = chronic otitis media; ETS = environmental tobacco smoke; NPA = nasopharyngeal aspirate; NTHi = non-typeable *Haemophilus influenzae*; OM = otitis media; URTI = upper respiratory tract infection;

Table 3.2 Experimental groups

	Non-COM prone control group	**COM prone group**	**Samples**
Immune cell Proportional Analysis	20 participants	20 participants	Blood Adenoid
Clinical Microbiology			Adenoid Nasal Aspirate
Humoral Response			Plasma Saliva
Demographics			Questionnaire Clinical Records

3.1.1 Study setting

The Rockhampton region of Central Queensland is a subtropical region on the east coast of Australia, approximately 600 kilometres north of the Queensland capital, Brisbane, with its main industries being agriculture, mining and power generation (Queensland Government 2014). The current population of the Rockhampton region is just under 115,500 residents, of whom 5.5 percent (5.5%) identify as ATSI people (Australian Bureau of Statistics 2014b).

The Rockhampton area of regional Queensland provided the infrastructure required to support the research, including established respiratory infectious disease research facilities within CQUniversity, collaborations with an ENT physician and private clinic at the Mater Hospital Rockhampton, a pathologist at Sullivan Nicolaides Pathology, Rockhampton and research partners at the Institute of Health and Biomedical Innovation at the QUT in Brisbane. The region was therefore well equipped to support OM clinical research; the first study of its kind in Queensland.

3.1.2 Participant sample size and demographics

This study is compliant with the National Statement on Ethical Conduct in Human Research 2007 and the Values and Ethics: *Guidelines for Ethical Conduct in Aboriginal and Torres Strait Islander Health Research 2003*. The study is governed by the CQUniversity and Mater Medical Research Institute HREC and the Mater Research Governance Office.

The study was a cohort-comparison study. The matching criterion for the cohorts was age, with children selected for each cohort between 2 and 7 years of age. The study did not require gender balance or bias, therefore the gender ratio was random to reflect the target population. In order to study local and systemic lymphocyte immunity involved with COM in this population, a localised (adenoid) and systemic (blood) source of lymphocytes was obtained from children who were suffering from COM, and from control children non-prone to COM. To obtain lymphocytes from the COM prone and non-COM prone populations, children between 2 and 7 years of age who were undergoing adenoidectomy and/or tonsillectomy for clinical reasons were recruited into this study following informed parental consent (see Appendix C). Group 1 was non-COM prone; the participants in this group were recruited from children who underwent adenoidectomy for clinical reasons other than COM infection, such as AH and/or tonsillitis. Group 2 was COM prone; the participants in this group were recruited from children who underwent adenoidectomy for clinical reason that were related to COM, such as chronic suppurative OM (CSOM) or rAOM. COM was defined as persistent COME for 3 months or more, or 3 episodes of AOM in 6 months, or 4 episodes of AOM in 12 months. Participant exclusion criteria included younger than 2 years of age or older than 7 years of age, craniofacial problems such as cleft palate, inborn or acquired immune deficiency, and known immunological impairment.

A power analysis was employed to calculate a sample size for each group that could determine a difference in effect in adenoidal T_{reg} lymphocytes between COM prone and non-COM prone groups. The power analysis was only done for the first primary outcome, where it was calculated based on published differences in T_{reg} lymphocyte populations between pneumococcal positive and negative children. Consultation with a Biostatistician revealed that hundreds of participants would be required to give the study enough power to find differences, if any, in demographic factors in COM prone and non-COM prone children. Given the time and resources allocated to the project, it was decided that a study population of hundreds was not feasible for this project and that the sample size should be restricted to meet the primary outcome of the study. The factors considered were a *p* value of 0.05, power of 80% and a population effect size of 1, based on the difference in T_{reg} lymphocyte percentage means available in published literature (Zhang et al. 2011). Using these parameters it was calculated that 16 participants were required in each group of the study, which agreed with the estimate provided by the nomogram in the sample size calculation review published by Whitley & Ball (2002). As the power analysis determined

that at least 16 participants were required in each group for the study to have good statistical power, 20 participants were allocated to each group to allow a suitable buffer for withdrawal rates, while still maintaining the overall necessary sample size.

3.1.3 Bacterial cultures and human tissues

S. pneumoniae invasive serotypes 14 and 18C, and commensal serotypes 6B and 19F were used in this study. These bacterial strains were chosen based on their clinical relevance to OM, inclusion in the PCV-13 vaccine, availability to the CCMI laboratory, and the prior use of these strains in *in vivo*, *ex vivo* and *in vitro* respiratory infection models in the CCMI laboratory and others (Lipsitch et al. 2000; Krishnamurthy et al. 2009). The adenoid, blood, nasopharyngeal aspirates and saliva samples were collected from the participants in the study, with parental consent and under ethical consideration. All dealings with participants in this study were subject to and monitored by the relevant HREC of CQUniversity and the Mater Hospital.

3.2 Collection of Tissue

The adenoids were removed surgically from participants via the curette method and transferred immediately into labelled sterile 120 ml tubes (Sarstedt) containing 50 ml of Processing media (RPMI 1640 supplemented with 100 U per ml penicillin, 100 U per ml streptomycin, 0.25 μg per ml amphotericin B, 200 mM L-glutamine and 2% heat-inactivated fetal calf serum (HI-FCS) (Life Technologies)) (Zhang et al. 2011). The blood was collected from the participants via venipuncture into labelled sterile ethylenediaminetetraacetic acid (EDTA) anti-clotting tubes (Becton Dickinson) that were sealed in snap-lock bags. Salivette tubes (Sarstedt) were used to collect saliva samples and nasal aspirates were collected via the saline syringe nasal aspirate method (BD Diagnostics 2005). The nasal aspirates and biopsies of the adenoid tissue were placed on ice, ready for transport to the laboratories of Sullivan Nicolaides Pathology, Rockhampton. The tubes containing the tissues and blood were placed immediately on ice and transported to the CQUniversity laboratories within 2 hours from time of collection. The Salivette tubes containing the saliva samples were centrifuged at 1000 × g for 2 minutes for harvest of the full saliva sample. Processed saliva was stored at -20°C until needed for the *S. pneumoniae*-specific IgA analysis. All sample tubes were labelled with a participant laboratory code (PLC) to enable de-identified (coded) identification of samples to match de-identified participant clinical data that was transferred from the clinician to the researcher via the case record form (see Appendix B).

3.3 Cell Culture Procedures

All cell cultures were prepared in a class II biohazard cabinet, situated in a designated sterile room within the laboratory. The operator wore appropriate protective clothing including a laboratory gown, gloves, eye protection, face mask and closed in shoes. All surfaces were decontaminated with 10% bleach solution and sterilized with 80% ethanol prior to and post all procedures. All equipment used was sterile and cell culture treated and aseptic technique was routinely employed.

3.3.1 Isolation of mononuclear cells from adenoids and peripheral blood

For processing of the adenoid, in a 40 x 11 mm sterile tissue culture dish (Techno Plastic Products - TPP), any grossly inflamed or necrotic tissue was removed from the adenoid sample. Using a sterile, disposable size 22 scalpel blade (Livingstone), the tissue was cut into small pieces in processing media supplemented with Deoxyribonuclease (DNAse) 1 (Sigma-Aldrich). To generate a cell suspension, the small pieces of tissue were processed gently through a sterile, stainless steel cell dissociation sieve (Sigma-Aldrich) that had been stacked on top of a fresh, sterile 40 x 11 mm tissue culture dish. Using a sterile 10 ml syringe plunger (Livingstone), the tissue was ground gently in the sieve, allowing the cells to strain through and collect into the dish. These were processed further into a single cell suspension through a nylon mesh 40 µm cell strainer (VWR) and collected into a fresh sterile 50 ml tube (Sarstedt), using the 10 ml syringe plunger and processing media. The cell suspension was made up to 10 ml with processing media and then transferred to a sterile 10 ml tube. The cell suspension remained undisturbed for 5 minutes to allow for sedimentation of tissue debris which was then removed.

In order to isolate the mononuclear cells (MNC) from the adenoid cell suspensions, the Ficoll method was used (GE Healthcare 2014). For each sample two sterile 10 ml tubes (Sarstedt) containing 3 ml of Ficoll-Paque PLUS (GE Healthcare Life Sciences) were prepared. Half of each sample (5 mL) was carefully layered over the Ficoll-Paque PLUS in each tube, bringing both tubes to a total volume of 8 ml each. To layer the cell suspensions over the Ficoll-Paque PLUS, the tube was carefully tilted on an angle and using a sterile transfer pipette a single drop of the sample was dispersed across the surface of the Ficoll-Paque PLUS and then added slowly to layer over the top, being careful to create a layered effect as opposed to a mixture. The tubes were then centrifuged at 400 × g, for 30 minutes at room temperature with no brake (deceleration set at zero). Using a sterile transfer pipette the top coat was collected and discarded. This exposed the buffy layer containing the

lymphocytes, which was carefully collected with a sterile pipette and transferred to a fresh sterile 10 ml tube. The lymphocytes were topped up to 10 ml with processing media and washed twice in a centrifuge at 600 × g for 10 minutes, with the brake on. Exactly half of the lymphocyte cell suspension (5 ml) was then layered over 2 × 10 ml sterile tubes each containing 3 ml of Ficoll-Paque PLUS.

For each 10 ml blood sample, the aliquot was diluted 1:1 using Dulbecco's phosphate buffered saline (dPBS) (Life Sciences), bringing the total volume of diluted blood to 20 ml. In 5 ml volumes, the diluted blood was layered over 3 ml of Ficoll-Paque PLUS in 10 ml tubes, using the same technique described for the adenoid cell suspension samples. All samples on Ficoll-Paque PLUS were centrifuged at 400 × g, for 30 minutes with no brake (deceleration set at zero).

For isolation of the lymphocytes, the top layer of each adenoid sample Ficoll separation was carefully collected and discarded, exposing the clean buffy coat that was collected carefully into a fresh sterile 10 ml tube using a sterile transfer pipette. Likewise, the plasma top layer of each blood sample Ficoll separation was collected carefully with a transfer pipette and placed into a fresh, sterile 10 ml labelled tube and stored at -80°C. The exposed buffy coat was carefully collected from the blood samples and transferred to a fresh sterile 10 ml tube. The adenoid MNC (AdMNC) and peripheral blood MNC (PBMC) were brought up to 10 ml volumes with processing media or dPBS respectively and washed twice with centrifugation at 100 × g for 10 minutes with the brake on. Each supernatant was removed and the cell pellet was resuspended in 2 ml of processing medium and placed on ice.

3.3.2 MNC viability count using haemocytometer method

From both the AdMNC and PBMC suspensions, a 10 µl aliquot was removed and mixed 1:1 with trypan blue (0.2% weight per volume), sodium chloride (Sahin-Yilmaz & Naclerio) and dPBS solution (200 µl of trypan blue mixed with 50 µl of 5 × saline (4.25% sodium chloride (NaCl)), and 200 µl of dPBS). Using a haemocytometer, 10 µl of the cell/trypan blue solutions were counted and the viable cell counts and total cell counts were recorder. To calculate the total number of viable cells as a concentration in the 2 ml AdMNC and PBMC suspensions, the number of viable cells in 25 squares was multiplied by 2 (dilution factor) and this value was then multiplied by 10 000 (area of the grid on haemocytometer). This gave the total number of viable cells per ml. This value of total

viable cells was then used to calculate the AdMNC and PBMC suspensions by scaling down to a desired concentration. This method was used to confirm AdMNC and PBMC viability to be greater than 95% following isolation, and greater than 91% following overnight resting of the isolated lymphocyte suspensions.

3.4 Immune Cell Proportional Analysis

Surveillance of immune cell types in the adenoids and blood was achieved by staining for cell markers and further analysis with flow cytometry. The immune cells investigated with their corresponding protein markers and fluorophore labels are listed in Table 3.3.

Table 3.3 Lymphocyte analysis

Lymphocyte	**Protein Marker**	**Measured as a percentage of**	**Fluorophore (Becton Dickinson)**
B lymphocyte	$CD19^+$	Total lymphocytes	Fluorescein isothiocyanate (FITC)
T lymphocyte	$CD3^+$	Total lymphocytes	Phycoerythrin-cyanine dye Cy7 (PE-Cy7)
Cytotoxic lymphocyte (T_C)	$CD3^+CD8^+$	Total lymphocytes	PE-Cy7 & BD Horizon V500 (HRZN V500)
T Helper lymphocyte (T_H)	$CD3^+CD4^+$	Total lymphocytes	PE-Cy7 & PerCP-cyanine dye Cy5.5 (PerCP-Cy5.5)
T regulatory lymphocyte (T_{reg})	$CD3^+CD4^+CD25^{high+}CD127^{low+}FoxP3^+$	T_H lymphocytes	PE-Cy7, PerCPCy5.5, BD Horizon Brilliant Violet 421 (BV421), Phycoerythrin-CF594 (PE-CF594) & Alexa Fluor 647 (AF647)

3.4.1 Cell marker staining for lymphocyte analysis using flow cytometry

For flow cytometry cell surface staining, the AdMNC and PBMC were isolated as described above. After resting the cells overnight in culture at approximately 1×10^6 cells per ml, in a humidified incubator at 37°C with 5% carbon dioxide (CO_2), the cells were collected from the culture flasks and transferred to sterile 10 ml tubes and made up to 10

ml volumes with dPBS. A 10 µl aliquot of cells for both the AdMNC and PBMC was removed for a post-overnight rest viability stain according to the methods described above using the haemocytometer. The cells were washed 3 times by centrifugation at 400 × g for 5 minutes with the brake on, with cell pellets being resuspended in fresh dPBS prior to each wash. The supernatants were discarded and the cell pellets were resuspended in the required volume of dPBS to bring them to a final concentration of 2×10^7 cells per ml. In aliquots of 100 µl cells were added as triplicates (duplicate tests for cell staining and a single unstained test) to 96 well U-bottom assay plates (Sarstedt), resulting in approximately 1×10^6 cells per 100 µl per well. All work from this point was completed on the laboratory bench as there was no requirement to maintain sterility in the remainder of the assay. At this point, cells were ready for staining.

Fixable viability dye eFluor 780 (Affymetrix eBioscience) was diluted 1/500 in dPBS and 100 µl was added to each well containing 100 µl of cells for staining, bringing the final live/dead stain down to a 1/1000 dilution. The cell suspensions were mixed by pipette and incubated in the dark at 4°C for 30 minutes. The plates were centrifuged at 400 × g for 5 minutes at 4°C to pellet the cells out of the stain. The supernatant was removed from the wells by hand, using a firm flick of the plate, and the cells were washed once by adding gently 200 µl of cell staining buffer (1 × dPBS supplemented with 2% HI-FCS) to the cells, pipette mixing and centrifuging at 400 × g for 5 minutes at 4°C. The supernatant was removed from the wells via a firm flick of the plate.

In order to identify the cell surface markers for the B and T lymphocytes subsets, cells were stained with fluorescent labelled mouse anti-human antibodies specific to CD19, CD3, CD4, CD8, CD25 and CD127 (Becton Dickinson), as described in Table 3.3. During the flow cytometry 8-colour panel optimisation, the cells were set up in control wells for florescence minus one (FMO) compensation controls and all fluorescent labelled antibodies were added to these wells, minus the fluorescent labelled antibody that was being controlled for as displayed in Table 3.4. A calculated dilution of each antibody (determined by cell concentration and titration of antibody) was used for the surface staining of cells. For CD19, CD3, CD8, CD25 and CD127 antibodies, a 1/25 dilution was used and for CD4 antibody a 1/20 dilution was used to surface stain cells. For a 50 µl assay volume, 25 µl of staining buffer was added to each test well and 50 µl staining buffer was added to wells for unstained cells. All surface staining antibodies were prepared in a multi-mix in staining buffer, where the required volume of each antibody was added to the mix to create a 2 ×

concentration. This resulted in a 1/12.5 dilution of CD19, CD3, CD8, CD25 and CD127, and 1/10 dilution of CD4. The surface staining mix was added to each test well of cells in 25 µl volumes to give a total of 50 µl in the test wells with the appropriate final dilutions of 1/25 and 1/20 dilutions reached, respectively. Plates were incubated for 30 minutes at 4°C, in the dark.

Table 3.4 FMO compensation controls

Antibody/ Stain for:	FMO CD3	FMO CD4	FMO CD8	FMO CD19	FMO CD25	FMO CD127	FMO FoxP3	FMO Live/Dead
CD3	×	✓	✓	✓	✓	✓	✓	✓
CD4	✓	×	✓	✓	✓	✓	✓	✓
CD8	✓	✓	×	✓	✓	✓	✓	✓
CD19	✓	✓	✓	×	✓	✓	✓	✓
CD25	✓	✓	✓	✓	×	✓	✓	✓
CD127	✓	✓	✓	✓	✓	×	✓	✓
FoxP3	✓	✓	✓	✓	✓	✓	×	✓
Live/dead	✓	✓	✓	✓	✓	✓	✓	×

Following surface staining antibody incubations, 50 µl of staining buffer was added to the test and unstained wells and the plates were centrifuged at 400 × g for 5 minutes at 4°C. The wash was removed from the wells via a firm flick of the plate. The cells were washed once more following the same protocol, using a 100 µl staining buffer wash volume. For permeabilisation and fixation of the cells 50 µl of fixation-permeabilisation 1 × buffer from the transcription factor buffer set (Becton Dickinson) was added to all wells and incubated for 45 minutes, at 4°C in the dark. 50 µl of permeabilisation-wash 1 × buffer from the transcription factor buffer set was added to the test and unstained wells to wash the cells as described above and a second wash was performed using 100 µl of permeabilisation-wash 1 × buffer. Unstained cells and stained test cells were resuspended in 50 µl and 40 µl of permeabilisation-wash 1 × buffer, respectively. To create a final 1/5 dilution of FoxP3 antibody, 10 µl of FoxP3 antibody was added to wells with stained cells and thoroughly pipette mixed, prior to incubating for 30 minutes at 4°C in the dark. To wash the cells out of the intracellular FoxP3 stain, 50 µl of permeabilisation-wash 1 × buffer was added to the wells and subject to wash procedures described above. A second wash was performed using 100 µl of permeabilisation-wash 1 × buffer. Finally, the cells were resuspended in 150 µl of staining buffer and transferred to labelled 1.5 ml tubes containing 150 µl of staining buffer, bringing the final volume of each cell suspension to 300 µl.

The labelled tubes containing the cells were stored in a plastic box within a cooler bag lined with ice packs for transport from CQUniversity, Rockhampton campus, to the Institute of Health and Biomedical Innovation Cell Imaging Facility at the QUT, Kelvin Grove campus, Brisbane. All cell suspensions were then stored refrigerated at 4°C overnight prior to cell acquisition and analysis on the FACSAria III (Becton Dickinson) the day following cell staining. Using the Diva software (Becton Dickinson), the lymphocytes were gated based on their size and granularity. To ensure only viable lymphocytes were included in the analysis, further gating was applied based on the fixable viability dye eFluor 780 (Affymetrix eBioscience). Using this strategy, it was consistently confirmed that greater than 91% of lymphocytes in suspension were viable at the time of staining and fixation, and were therefore included in the analysis. Further gating analysis was applied to these gated lymphocytes through detection of the fluorophore labelled cell markers, and lymphocyte subset percentages were obtained based on the cell markers outlined in Table 3.3.

3.5 Microbiological Procedures

3.5.1 Clinical microbiological analysis

Adenoid biopsies and nasal aspirates were sent from the Mater Hospital Rockhampton with the routine pathology collections to the laboratories of Sullivan Nicolaides Pathology, Brisbane. Upon receipt, samples were tested for growth of *S. pneumoniae*, *M. catarrhalis*, NTHi, *S. aureus*, *Streptococcus pyogenes*, Group A Streptococcus, *Alloiococcus otitidis* and *Pseudomonas aeruginosa*. Detection was initially documented as negative, light, moderate or heavy positive growth. To simplify the analysis and present data in a format comparable to other studies, these level of growth sub-results were consolidated within major groupings of positive or negative culture for each bacterial species, presented in Chapter 5, Table 5.1. For other organisms detected, the bacterial species was identified and the degree of growth was noted.

All specimens were cultured in CO_2 and anaerobic conditions. Specifically for *S. pneumoniae* grown on blood agar plates, alpha haemolytic concave colonies, 1 to 2 mm in size, were grown on a 16 streak plate with an optochin disc. If the organism was sensitive it was identified as *S. pneumoniae*. For organisms that were optochin resistant, but still appeared morphologically as *S. pneumoniae*, the automated VITEK system was used for identification. For *M. catarrhalis*, 2 to 3 mm colonies were grown on chocolate agar and

then tested with oxidase and indoxyl acetate. If the organism was positive for these tests and identified as a gram negative cocci in the gram stain, it was identified as *M. catarrhalis*. If the organism was an oxidase positive, gram negative cocci, but indoxyl acetate negative, the automated VITEK system was used for identification. For NTHi, 2 to 3 mm colonies that grew better on chocolate agar compared to blood agar were tested for growth with factors XV, V and X. Organisms that grew only with the XV factor combination were identified as NTHi. Organisms that grew on chocolate agar, yet failed to grow with the factors were identified using the automated VITEK system. For *S. aureus*, 2 to 3 mm colonies with a cream/yellow appearance on agar plates were tested for agglutination on Staph latex, and if positive they were identified as *S. aureus*. If the Staph latex test was negative, but *S. aureus* was still suspected, the automated VITEK system was used for identification. For *S. pyogenes* (Group A Streptococcus), 2 to 3 mm, beta haemolytic colonies grown on blood agar were tested for agglutination on a Streptococcus latex test, and if positive were identified as *S. pyogenes* (Group A Streptococcus). For *P. aeruginosa*, 3 to 4 mm colonies with fuzzy edges, grown on MacConkey agar, and were oxidase positive, were then grown on cetrimide agar. If the organisms produced a green to brown pigment on the cetrimide agar they were identified as *P. aeruginosa*. If an organism failed to produce the pigment on the cetrimide agar, the automated VITEK system was used for identification. For *A. otitidis*, following 3 days of culture on agar plates, colonies approximately 1 mm in size with a grey appearance were identified as *A. otitidis* using the automated VITEK system.

It is considered that gaining a more sensitive screening of the nasopharyngeal colonisation, perhaps through polymerase chain reaction (PCR) methods, would influence the microbiological results. Although PCR is recognised as a more sensitive method for microbial detection, it too has limitations including the spectrum of nasopharyngeal pathogens which it can detect (Eser et al. 2009). In the present study, only live bacterial cultures of the nasopharynx were of interest, where eight species were included in the screening, with other cultures documented. The findings were also intended to have translational relevance to physicians, for whom clinical cultures are the preferred method for microbiological diagnosis. Therefore, based on these factors, the study design included the use of conventional culture methods rather than PCR for the clinical microbiology assessment.

3.5.2 Recovery and maintenance of S. pneumoniae

Sterile Columbia agar plates (Micromedia) were used to revive and subculture *S. pneumoniae*. A sterile inoculum loop (Sarstedt) was used to recover the bacteria from the frozen stock tubes and streak onto the appropriate agar plates. The plates were incubated for 16 hours overnight in a humidified incubator at 37°C with 5% CO_2. The bacteria were sub-cultured twice on consecutive days to establish good growth and viability.

3.5.3 Whole killed cell preparations of S. pneumoniae

In order to prepare a whole killed cell (WKC) suspension of the bacteria, live serotypes of *S. pneumoniae* were prepared to a known concentration (approximately 1×10^{10} colony forming units (CFU) per ml). This was achieved via the growth of bacterial lawns. *S. pneumoniae* lawns were grown on 5 × Columbia-gentamicin agar plates (Micromedia) per serotype. Sterile glass slides and sterile PBS were used to harvest the bacteria from the agar plates into sterile 10 ml collection tubes per serotype. The live bacterial suspensions were made up to a volume of 10 ml with PBS and centrifuged to a pellet at 2800 × g for 5 minutes at room temperature. The bacterial pellets were resuspended in 10 ml of 70% ethanol and incubated at 37°C with humidity and 5% CO_2 for 3 hours in a rotating mixer, to kill the bacteria. The WKC suspensions were then washed 3 times in sterile dPBS with centrifugation at 2800 × g at room temperature, for 5 minutes. Following the final wash, the WKC pellets were resuspended in 1 ml of sterile dPBS.

The 4 *S. pneumoniae* serotypes were combined into a single 10 ml tube to give a *S. pneumonaie* mix with a total volume of 4 ml. For the antigen to be prepared appropriately for detection by plasma and saliva-derived Igs, the WKC *S. pneumoniae* mix required further processing by sonication. On ice, the WKC suspension was transferred to an appropriately labelled tube and placed in a -20°C freezer for long term storage. For quality control the suspension of *S. pneumoniae* WKC was streaked onto Columbia agar plates and incubated overnight at 37°C with humidity and 5% CO_2, to check for the growth of any viable bacteria.

3.5.4 Preparations of S. pneumoniae WCSA

For the antigen to be prepared appropriately for detection by plasma and saliva-derived Igs in ELISAs, the WKC *S. pneumoniae* mix needed to be further processed by sonication. On ice, the WKC bacterial suspension was subject to pulse sonication. Using the microtip on the Model 3000 Ultrasonic Homogeniser (BioLogics Inc.) the instrument was set to 50%

power and 1 minute of 4 second sonication pulses. Care was taken to ensure the WKC suspension did not foam or heat up, as this would increase the risk of protein denaturation. The *S. pneumoniae* WCSA sample was diluted by a factor of 1/10 using milli Q water and the absorbance at 280 nm, 260 nm and 320 nm was measured in 1 cm light path uVettes (Eppendorf) in the Eppendorf Biophotometer Plus (Eppendorf). Using the Warburg formula, A260 nm and A280 nm readings, the protein concentration for the *S. pneumoniae* WCSA sample was calculated (Vogel et al. 2004). For quality control the WCSA suspension was streaked onto Columbia agar plates and incubated overnight at 37°C with humidity and 5% CO_2, to check for the growth of any viable bacteria. The *S. pneumoniae* WCSA was labelled accordingly with the serotype mix, protein concentration and date, and was stored in a -20°C freezer until required for use in the ELISAs.

3.6 *S. pneumoniae*-specific Ig Detection

The measurement of *S. pneumoniae*-specific total IgG and total IgA in plasma and total IgA in saliva samples from the enrolled participants was performed using the standard sandwich ELISA method following manufacturers instruction (Affymetrix eBioscience), with 100 µl assay volume. All assay buffers and antibodies were supplied from the Human IgG or IgA Total Ready-Set-Go! ELISA kits (Affymetrix eBioscience) unless otherwise stated. The *S. pneumoniae* WCSA mentioned previously was diluted to 1 µg per ml in 1 × coating buffer. The *S. pneumoniae* WCSA was coated onto sample and blank wells of 96 well Polysorp ELISA plates (Nunc). Either anti-human IgG or IgA capture antibodies were diluted in 1 × coating buffer according to the kit instructions and these were added to corresponding standard curve wells. The coated plates were incubated overnight at 4°C in the dark.

Following the coating incubation all plates were washed 4 times in wash buffer (1 part 10 × PBS pH 6.6, 9 parts milli Q water and 0.05 parts Tween 20) (Sigma-Aldrich). To all wells, 250 µl of 2 × assay buffer A (block solution) was added and the plates were incubated at room temperature, in the dark, for 2 hours. For all wells coated in *S. pneumoniae* WCSA, the plasma samples were diluted in 1 × assay buffer A to a final 1/1000 dilution in the wells. The saliva samples were diluted in 1 × assay buffer A to a final 1/500 dilution for all *S. pneumoniae* WCSA coated wells. Plasma or saliva samples were added to all test wells. To the standard curve wells, purified human IgA or IgG antibodies were diluted down to 100 ng per ml in 1 × coating buffer and diluted serially by a factor of 2 down the plate in duplicates until a final concentration of 1.5625 ng per ml was reached. The 1 × assay buffer

A was added to blank wells and all the plates were incubated at room temperature, in the dark, for 2 hours. The plates were washed 4 times in wash buffer, anti-human IgA or IgG horse radish peroxidase (HRP)-labelled detection antibody was added to all wells and the plates were incubated in the dark, at room temperature, for 2 hours. Another 4 washes were performed with wash buffer, then tetramethylbenzidine (TMB) substrate solution was added to all wells and incubated at room temperature, in the dark, for 15 minutes. The reaction was stopped by the addition of 0.5 M sulphuric acid to all wells. The chemiluminescent signal from each well was measured at 450 nm on an optical density plate reader. IgA and IgG standard curves were generated from the absorbance measures of the known IgA and IgG standard concentrations and the sample well absorbance measures were plotted against the relative standard curve to determine the *S. pneumoniae*-specific total IgA and total IgG protein concentrations in plasma and or saliva samples, respectively.

3.7 Statistical Analysis

Data sets were analysed using IBM SPSS Statistics version 22 software. Groups were defined by the study cohorts, demographic and environmental factors, microbial culture and immunology factors (COM prone versus non-COM prone; culture positive versus culture negative, male versus female, etc.) and were used for comparison within tests. To compare groups of interest and identify significant risk factors, the statistical tests that were performed include descriptive and frequencies analyses, chi-square tests with continuity correction, Fisher's Exact tests, Spearman correlation analyses, logistic regressions, independent student *t*-tests, one and two way analysis of variance (ANOVA)s and non-parametric Mann-Whitney U tests. The Fisher's exact test of independence was used when cell values in the SPSS chi-square tables had an expected frequency of five or less. All statistical tests were chosen dependent on the type (continuous, categorical or nominal) of independent and dependent variables that were included in the test, the number of variables included in the test, the sample number included in the test, and the outcome that was sought (Pallant 2013). A p value of less than or equal to 0.05 was used to define statistical significance. Antibiotic therapy within the previous 6 months was recognised as a confounding variable in the analysis of microbiological data, therefore where necessary it was included as a confounding variable in the microbial analysis.

4 COMPARATIVE ANALYSIS OF DEMOGRAPHIC FACTORS WITH INFECTION AND IMMUNITY IN CHRONIC OTITIS MEDIA

4.1 Introduction

OM is primarily a paediatric disease that exists as a global health issue, causing hearing, speech, learning and social impairments, loss of income, and costs to government healthcare in the order of billions of dollars (Klein 2001). Within Australia OM greatly affects Indigenous children with more than 90 percent affected by the disease before reaching two years of age (Morris et al. 2005). Overall, it is recognised as a common respiratory illness in Australia with nearly 50 percent of non-Indigenous Australian children also experiencing an episode of OM prior to two years of age (Jacoby et al. 2008). Of what is understood of OM in Australia, much is in relation to remote communities within western and northern parts of the country, whereas very little is understood of the disease on the east coast.

The causative conditions which give rise to OM are multi-factorial involving microbial interactions, host immunity, and both demographic and environmental risk factors (Daly et al. 2010). In understanding the aetiology of OM, and given the complexity of the host and its microbial ecology, the interactions of several causative factors are usually what will predispose a child to OM (Lehmann et al. 2008). This is demonstrated well in the literature where graphics and reviews are extensive in outlining the complex paradigm that is OM, as aetiological factors include but are not limited to, microbial-host interactions, bacterial carriage, age, male gender, ETS exposure, child care attendance, socioeconomic status and seasonality. However, the causal pathways all diverge into four distinct groups, these being microbial, host, demographic and environmental (Lehmann et al. 2008; Daly et al. 2010).

Generally, males are more prone to OM than are females. COM also occurs most commonly during preschool years and occurs early in life, when AOM is the most common form of OM in infancy (Daly et al. 1999). This factor is one of the strongest determinants for COM in early childhood (Teele et al. 1989; Daly & Giebink 2000). Factors in household and childcare arrangements are potential risks for COM including overcrowding, having older siblings, having siblings with a history of OM, and attending communal childcare (Zielhuis et al. 1989; Uhari et al. 1996; Lamphear et al. 1997; Rovers et al. 2004).

Environmental factors may contribute to a child developing COM. There is a lack of consensus around whether or not exposure to tobacco smoke has an impact on the outcome of OM development. The conclusions in the literature for children 2 years and older are contradictory on whether it is or is not identified as a risk factor (Zielhuis et al. 1989; Stenstrom et al. 1993; Lamphear et al. 1997; Caylan et al. 2006; Sophia et al. 2010; Martines et al. 2011).

Early colonisation events with *S. pneumoniae*, *M. catarrhalis* and NTHi have also been linked to the increased risk of developing OM, thereby contributing to the aetiology of the childhood disease (Leach et al. 1994; Faden et al. 1997). Although the causative pathogens are well defined, their association with the demographic and environmental factors that contribute to COM are less well understood.

Surprisingly little is known of the association of demographic and environmental OM risk factors with lymphocyte distributions in children. Of all the risk factors described herein, age is understood most in regard to its associations with blood and adenoid lymphocyte populations in children. It is known that with increasing age T, T_C and T_H lymphocytes in the adenoids do not differ in their proportions between children who have OM and those without OM. Children who have OM show a decline in B lymphocytes from the adenoid with increasing age (Hemlin et al. 1995; Mattila & Tarkkanen 1997; Lagging et al. 1998). The levels of B lymphocytes and T_H lymphocytes in the blood also decline in children as they grow older (Osugi et al. 1995; Comans-Bitter et al. 1997). Factors including gender and exposure to tobacco smoke have been investigated minimally for their associations with blood and adenoid-derived lymphocytes in children, yet the results are inconclusive due to differences in the ages of the study populations, and how well passive cigarette smoke exposure was defined (Lee et al. 1996; Lisse et al. 1997; Avanzini et al. 2006; Vardavas et al. 2010). There was a single study that reported no associations between a child being the eldest, middle or youngest born and the percentage of lymphocyte subsets in the blood (Lisse et al. 1997). Further to this, there are several gaps in the published literature for understanding the associations between other mentioned demographic and environmental risk factors of OM with adenoid and blood lymphocyte populations in children, particularly in relation to the T_{reg} lymphocytes. T_{reg} lymphocytes are a subset of T_H lymphocytes that contribute to the regulation of immune responses to antigen, by way of immunosuppression; their immunosuppressive responses to commensal bacteria induce immune tolerance, sustained colonisation at mucosal sites, and contribute to the

progression of infection and chronic disease (Jang 2010). The association of T_{reg} lymphocytes with *S. pneumoniae* colonisation has been investigated, although their relationship with COM and other factors associated with COM remains largely unknown (Pido-Lopez et al. 2011; Zhang et al. 2011).

Although it is understood that these interactions are important in causing OM, there is much to be learnt about the mechanisms of such interactions. Furthermore, the true relationships among the microbial, host, demographic and environmental factors are poorly understood. This study aims to determine what relationships exist among these factors, what environmental and demographic factors are associated with bacterial nasopharyngeal carriage and an increased risk of COM, and how the environmental and demographic factors are associated with lymphocyte subset proportions, including the T_{reg} lymphocytes, in COM and non-COM prone children. The results from this chapter will contribute to understanding how demographic and environmental factors of OM relate to commensal bacteria within the nasopharynx, and if they are positively associated with T_{reg} lymphocytes in the adenoids and blood of children with COM, thereby potentially contributing to host-tolerance of nasopharyngeal colonisation and the development of COM.

4.2 Results

4.2.1 Characteristics of study population

Enrolment of COM & non-COM prone children was completed in April 2014. Table 4.1 summarises the demographic characteristics of the study cohorts. There were slightly more males in the study with 60% of COM prone children and 65% of non-COM prone children being male, while 40% and 35% of children were female in the COM and non-COM prone groups, respectively. Children aged 2 to 3 years accounted for 50% of COM prone children and 55% of the non-COM prone children, while 50% of COM prone children and 45% of non-COM prone children were between 4 and 7 years of age. The mean age for COM prone children was 3 years 7 months and a standard deviation (SD) of 1.66. There were two children in the study who identified as ATSI, one of whom was COM prone and the second child was non-COM prone.

Table 4.1 Demographics of study cohorts

Demographic	Non-COM prone	COM prone	*p* value
Age			0.75
2 - 3 years	55 (11)	50 (10)	
4 - 7 years	45 (9)	50 (10)	
Sex			0.74
Male	65 (13)	60 (12)	
Female	35 (7)	40 (8)	
ATSI Heritage			0.76*
Yes	5 (1)	5 (1)	
No	95 (19)	95 (19)	
Number of children in household ≤15 years of age			0.75
≤ 2 children	60 (12)	55 (11)	
≥3 children	40 (8)	45 (9)	
Child's birth order			0.42
Youngest	45 (9)	35 (7)	
Middle	25 (5)	15 (3)	
Eldest	30 (6)	50 (10)	
Siblings with a history of OM			1.00
Yes	45 (9)	45 (9)	
No	55 (11)	55 (11)	
ETS exposure			0.29
Yes	35 (7)	20 (4)	
No	65 (13)	80 (16)	
Childcare			0.68
Yes	85 (17)	80 (16)	
No	15 (3)	20 (4)	
Immunisation compliance			0.50*
Yes	100 (20)	95 (19)	
Partial	0 (0)	5 (1)	
No	0 (0)	0 (0)	
History of URTI			0.17
Yes	60 (12)	80 (16)	
No	40 (8)	20 (4)	
Presence of AH			0.76*
Yes	95 (19)	95 (19)	
No	5 (1)	5 (1)	
Antibiotic therapy in previous 6 months			**0.02***
Yes	65 (13)	95 (19)	
No	35 (7)	5 (1)	
Steroid therapy in previous 6 months			0.50*
Yes	15 (3)	10 (2)	
No	85 (17)	90 (18)	

Values are presented as % (*n*). Values in bold indicate significance in *t*-test or chi-square analysis. * Fisher's exact test 1-tailed.

4.2.2 Environmental characteristics of study population

Table 4.1 summarises the environmental characteristics of the study cohorts. Only 45% of children prone to COM lived in a home housing three or more children, whereas the remaining 55% lived in a home that housed two children or less, therefore overcrowding was not associated with COM proneness. In COM prone children 50% were the eldest child, while the further 50% were the middle or youngest child, yet no significant differences were observed concerning the birth order of the child and the association with COM proneness. In both COM and non-COM prone children 45% had a sibling who had previously also been diagnosed with OM, therefore no differences were observed in the two groups concerning a sibling history of OM. Childcare, kindergarten, preschool or school attendance was similar between the COM prone and non-COM prone groups, as were ETS exposure and routine immunisation compliance.

4.2.3 Demographic, environmental and clinical risk factors associated with COM

To determine associations with demographic factors and a child being prone to COM, similar statistical methods where used to those described in other studies (Wiertsema et al. 2011). Demographic factors were cross tabulated with the outcome of COM prone and non-COM prone children in chi-square tests (significance reported for Fisher's exact test of independence for cells in the table with frequencies of 5 or less). Of all demographic factors, there was a significant difference where antibiotic therapy within the previous 6 months occurred more in COM prone children compared to non-COM prone children (see Table 4.1).

To determine the demographic factors that were predictors of a child being prone to COM, the demographic independent variables were compared individually with the outcome of COM prone using the univariate logistic regression analysis. For all demographic factors listed in Table 4.1, only antibiotic therapy had a significant effect with COM prone children, where the incidence of a child being prone to COM increased by 10 fold if they had antibiotic treatment within the previous 6 months (odds ratios (OR) = 10.23, 95% confidence interval (CI) = 1.12 – 93.34, $p = 0.04$). Other factors listed in Table 4.1 did not show a significant increased or decreased risk for a child being prone to COM in the univariate logistic regression analysis (see Appendix D. Table 9.1). To determine if antibiotic therapy was an independent predictor for the increased risk of a child being prone to COM, a multivariate logistic regression analysis was performed that included the demographic factors of interest. In this study, 4 to 7 year olds (OR = 2.37, CI = 0.39 –

14.32), ATSI status (OR = 6.31, CI = 0.08 – 500.71), a household with 3 or more children (OR = 2.99, CI = 0.30 – 29.78), and antibiotic therapy within the last 6 months (OR = 9.45, CI = 0.44 – 204.30) were all factors that had increased odds with a child being COM prone, however these factors were not significant independent predictors (see Table 4.2).

Other factors including males, a child's birth order, siblings with a history of OM, ETS exposure, childcare attendance, AH and steroid therapy within the last 6 months showed no evidence of being potential risk factors for a child being COM prone and were not statistically significant in their associations with the outcome of COM prone (see Table 4.2). There was a 27% risk of being prone to COM with having a history of URTI (OR = 1.27, CI = 0.12 – 13.40), although this was not significant (see Table 4.2).

Table 4.2 Binary logistic regression odds ratios & 95% confidence intervals predicting COM prone

Risk Factor	**COM (n=20) OR (95% CI)**	***p* value**
Age (2 - 3 years reference)**,** 4 - 7 years	2.37 (0.39 – 14.32)	0.35
Sex Male	0.47 (0.07 – 3.14)	0.43
ATSI	6.31 (0.08 – 500.71)	0.41
Number of children in household ≤15 years of age (≤ 2 children reference), ≥3 children	2.99 (0.30 – 29.78)	0.35
Child's birth order (Eldest reference) Middle Youngest	 0.10 (0.00 – 2.28) 0.35 (0.05 – 2.58)	 0.15 0.30
Siblings with a history of OM	1.00 (0.16 – 6.26)	1.00
ETS exposure	0.29 (0.03 – 2.51)	0.26
Childcare	0.59 (0.04 – 9.98)	0.72
History of URTI	1.27 (0.12 – 13.39)	0.84
Presence of AH	0.63 (0.01 – 55.41)	0.84
Antibiotic therapy within the last 6 months	9.45 (0.44 – 204.30)	0.15
Steroid therapy within the last 6 months	0.72 (0.04 – 14.71)	0.83

AH = adenoid hypertrophy; ATSI = Aboriginal and Torres Strait Islander; CI = confidence interval; COM = chronic otitis media; ETS = environmental tobacco smoke; OM = otitis media; OR = odds ratio; URTI = upper respiratory tract infection.

4.2.4 Demographic, environmental and clinical risk factors associated with OM pathogens

To determine associations and differences among demographic factors and nasopharyngeal colonisation, chi-square tests were performed; with cross-tabulations of the different demographic factors with different clinical microbiology groups (see Table 3.1). Clinical microbiology was categorised into the following groups; nasopharyngeal colonisation (any otopathogen culture irrespective of culture site), adenoid or nasal colonisation (any otopathogen culture in the adenoid biopsy or NPA respectively), multiple colonisation

(culture positive for two or more otopathogens irrespective of culture site), and *S. pneumoniae*, *M. catarrhalis*, NTHi, *S. aureus*, and other colonisation (species-specific culture irrespective of culture site). Gender, birth order and ETS exposure were all associated significantly with nasopharyngeal colonisation. Males had increased nasopharyngeal colonisation rates compared to females ($p = 0.04$). NTHi nasopharyngeal colonisation had a significant association with the youngest child among siblings, where NTHi colonisation was increased ($p = 0.04$). With ETS exposure there was increased *M. catarrhalis* colonisation ($p = 0.04$).

To determine if the demographic independent variables were determinants of colonisation, these were compared individually with the outcome of different nasopharyngeal colonisation groups using the univariate logistic regression analysis. Gender had a significant effect with nasopharyngeal colonisation and NPA colonisation, where the incidence of a child having positive otopathogen culture increased by approximately 10 fold if they were male (nasopharyngeal colonisation OR = 10.22, CI = 1.00 – 104.32, $p = 0.050$; NPA OR = 9.80, CI = 1.85 – 51.93, $p = 0.007$). When all demographic factors were considered in a multivariate logistic regression model, male gender was no longer an independent predictor of naopharyngeal or NPA positive otopathogen culture (see Table 4.3a). When considering the risk of the youngest child among siblings having positive NTHi or *S. aureus* nasopharyngeal culture, the univariate logistic regression model predicted a 15 fold increased risk of NTHi colonisation, and an 82% decreased risk for *S. aureus* colonisation. When adjusting for other demographic factors as possible predictors of NTHi or *S. aureus* colonisation including sex, history of URTI and ETS exposure, and controlling for antibiotics in the last 6 months in the multivariate logistic regression, the youngest child among siblings was confirmed as an independent determinant of NTHi and *S. aureus* nasopharyngeal colonisation (see Table 4.3b). The univariate logistic regression analyses demonstrated that ETS exposure increased the risk of *M. catarrhalis* and *S. aureus* nasopharyngeal culture, with the binary logistic regression analyses confirming the increased risk when adjusting for the youngest child among siblings as a determinant of *S. aureus* colonisation and antibiotics within the last 6 months as a confounding variable (see Table 4.3b). There were no associations evident between the number of children living in the household, childcare, kindergarten, preschool or school attendance, the children having siblings with a history of OM, URTI and the children's nasopharyngeal bacterial culture as defined by the various groups described above.

Table 4.3(a) Binary logistic regression odds ratios & 95% confidence intervals predicting nasopharyngeal colonisation

	Nasopharyngeal otopathogen positive culture		**Adenoid otopathogen positive culture**		**NPA otopathogen positive culture**		**Other otopathogen positive culture**		**Multiple otopathogen positive culture**	
Risk Factor	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 36, OR (95% CI), *p* value**	**Multivariate *n* = 36, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**
Age (continuous) 2 - 3 years vs. 4 - 7 years	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Sex Male vs. Female	**10.22 (1.00 – 104.32), 0.050**	ns	ns	ns	**9.80 (1.85 – 51.93),0.007**	ns	ns	ns	3.75 (0.89 – 15.81), 0.07	ns
Number of children in household ≤15 years of age ≤ 2 children vs. ≥3 children	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Child's birth order Youngest vs. Middle vs. Eldest	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Siblings with a history of OM	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
ETS exposure	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Childcare	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
History of URTI	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Antibiotics within the last 6 months	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Steroids within the last 6 months	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

CI = confidence interval; ETS = environmental tobacco smoke; NPA = nasopharyngeal aspirate; OM = otitis media; OR = odds ratio; URTI = upper respiratory tract infection. Values in bold indicate significance.

Table 4.3(b) Binary logistic regression odds ratios & 95% confidence intervals predicting otopathogen nasopharyngeal colonisation

	***S. pneumoniae* nasopharyngeal positive culture**		***M. catarrhalis* nasopharyngeal positive culture**		***NTHi* nasopharyngeal positive culture**		***S. aureus* nasopharyngeal positive culture**	
Risk Factor	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 36, OR (95% CI), *p* value**	**Multivariate *n* = 36, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**
Age (continuous) 2 - 3 years vs. 4 - 7 years	ns	ns	ns	ns	ns	ns	ns	ns
Sex Male vs. Female	ns	ns	ns	ns	4.65 (0.84 – 25.66), 0.078	ns	ns	ns
Number of children in household ≤15 years of age ≤ 2 children vs. ≥3 children	ns	ns	ns	ns	ns	ns	ns	ns
Child's birth order Youngest vs. Middle vs. Eldest	ns	ns	ns	ns	**15.75 (2.37 – 104.54), 0.004**	**17.32 (2.03 – 147.63), 0.009**	0.18, (0.30 – 1.10), 0.06	**0.01 (0.00 – 0.97), 0.048**
Siblings with a history of OM	ns	ns	ns	ns	ns	ns	ns	ns
ETS exposure	ns	ns	**5.75 (1.12 – 29.41), 0.04**	**9.04 (1.11 – 73.79), 0.04**	ns	ns	3.56 (0.79 – 16.14), 0.10	**44.40 (1.25 – 1583.81), 0.04**
Childcare	ns	ns	ns	ns	ns	ns	ns	ns
History of URTI	ns	ns	ns	ns	8.57 (0.95 – 77.01), 0.06	ns	ns	ns
Antibiotics within the last 6 months	ns	ns	ns	ns	ns	ns	ns	ns
Steroids within the last 6 months	ns	ns	ns	ns	ns	ns	ns	ns

CI = confidence interval; ETS = environmental tobacco smoke; NPA = nasopharyngeal aspirate; NTHi = non-typeable *H. influenzae*; OM = otitis media; OR = odds ratio; URTI = upper respiratory tract infection. Values in bold indicate significance.

4.2.5 Demographic, environmental and clinical risk factors associated with lymphocyte populations

Independent student *t*-tests, one way ANOVA and Mann Whitney U-tests were used to compare the differences and effects among the lymphocyte subsets of the blood and adenoid with demographic and clinical factors. Children aged 4 years or older had significantly reduced percentages of $CD4^+$ and $CD19^+$ lymphocytes in the blood ($CD4^+$ M = 36.4%, SD = 9.0%; $CD19^+$ M = 8.5%, SD = 3.2%); however the percentage of $FoxP3^+CD25^{hi+}CD127^{low+}$ lymphocytes were significantly increased (M = 4.9%, SD = 1.7%) compared to children aged 3 years or younger ($CD4^+$ M = 44.3%, SD = 6.7%; t (37) = 3.14, p = 0.003; $CD19^+$ M = 10.8%, SD = 3.8%; t (37) = 2.07, p = 0.045; $FoxP3^+CD25^{hi+}CD127^{low+}$ M = 3.7%, SD = 1.8%; t (37) = -2.07, p = 0.046). There were no relationships or differences observed with age and the distribution of other lymphocyte subsets in the blood or adenoid (see Appendix D, Table 9.2). Childcare attendance also had a negative association with $CD4^+$ lymphocytes in the blood, with these lymphocytes being significantly less for children who attended childcare (M = 38.6%, SD = 8.3%), compared to children who did not attend any form of childcare (M = 49.0%, SD = 5.1%; t (37) = 3.15, p = 0.003). There were no associations or differences observed with childcare attendance and the distribution of other lymphocyte subsets in the blood or adenoid (see Appendix D, Table 9.2). The youngest child among siblings had increased $CD4^+$ lymphocytes in the blood, however this difference was not statistically significant when compared to children who were the middle or eldest born child, and no other lymphocyte subsets in the blood or adenoids were significantly different when compared with the youngest, middle or eldest child among siblings (see Table 4.4).

Of children with siblings that had a history of OM, their $CD3^+$ and $CD8^+$ lymphocytes were significantly increased in the adenoid ($CD3^+$ M = 44.1%, SD = 8.5%; $CD8^+$ M = 7.9%, SD = 2.5%), although significantly decreased in their blood ($CD3^+$ M = 68.8%, SD = 8.4%; $CD8^+$ M = 23.0%, SD = 6.5%) compared to children who did not have siblings with a history of OM (adenoid-derived $CD3^+$ M = 36.8%, SD = 8.2%, t (36) = -2.69, p = 0.01; $CD8^+$ M = 6.1%, SD = 2.4, t (36) = -2.26, p = 0.03; blood-derived $CD3^+$ M = 74.7%, SD = 5.7%, t (37) = 2.61, p = 0.01; $CD8^+$ M = 27.6%, SD = 6.7%, t (37) = 2.16, p = 0.04). No other significant differences in the adenoid or blood-derived lymphocyte subsets were observed in children with or without siblings with a history of OM. $FoxP3^+CD25^{hi+}CD127^{low+}$ lymphocytes in the blood were not significantly different in

males (M = 4.7%, SD = 2.0%) compared to females (M = 3.5%, SD = 1.2%; t (37) = 2.00, p = 0.052). There were no observed differences with gender and the distribution of other lymphocyte subsets in the blood or adenoid. Furthermore, no differences were observed with the number of children in the household, ETS exposure, or URTI (inclusive of tonsillitis and/or OM) and the lymphocyte distributions in the adenoid (see Appendix D, Table 9.2) or blood.

Table 4.4 Independent student t-test values for differences & effect in blood lymphocyte subset percentages with demographic factors

Blood derived lymphocytes					
	B Lymphocytes	**T Lymphocytes**	**T_C lymphocytes**	**T_H lymphocytes**	**T_{reg} lymphocytes**
Risk Factor	***M*%, *SD*%, *p* values**	***M*%, *SD*%, *p* value**	***M*%, *SD*%, *p* value**	***M*%, *SD*%, *p* value**	***M*%, *SD*%, *p* value**
Age (continuous)					
2 - 3 years (*n* = 20)	**10.8, 3.8**	73.1, 6.5	24.3, 4.6	**44.3, 6.7**	**3.7, 1.8**
4 - 7 years (*n* = 19)	**8.5, 3.2, 0.045**	71.2, 8.5, 0.44	27.0, 8.7, 0.23	**36.4, 9.0, 0.003**	**4.9, 1.7, 0.046**
Sex					
Male (*n* = 25)	9.3, 4.0	71.1, 7.3	25.8, 7.2	39.5, 9.0	4.7, 2.0
Female (*n* = 14)	10.4, 3.2, 0.36	73.9, 7.8, 0.27	25.3, 6.7, 0.85	42.2, 8.5, 0.35	3.5, 1.2, 0.052
Number of children in household ≤15 years of age					
≤ 2 children (*n* = 22)	8.8, 2.9	74.1, 7.2	26.4, 7.3	41.1, 10.0	4.6, 1.8
≥3 children (*n* = 17)	10.8, 4.3, 0.11	69.6, 7.3, 0.06	24.6, 6.5, 0.44	39.6, 7.1, 0.62	3.9, 1.8, 0.27
Child's birth order					
Youngest (*n* = 15)	10.4, 4.5	73.6, 6.0	24.1, 6.1	43.9, 9.4	4.8, 2.2
Middle (*n* = 8)	10.5, 3.9	71.8, 6.1	26.5, 5.0	39.7, 3.6	4.3, 2.1
Eldest (*n* = 16)	8.6, 2.5, 0.87	70.9, 9.4, 0.53	26.5, 8.5, 0.46	37.6, 9.2, 0.81	3.8, 1.2, 0.88
Siblings with a history of OM					
Yes (*n* = 17)	10.2, 4.4	**68.8, 8.4**	**23.0, 6.5**	39.8, 10.1	4.1, 2.1
No (*n* = 22)	9.3, 3.1, 0.47	**74.7, 5.7, 0.01**	**27.6, 8.7, 0.04**	41.0, 7.8, 0.67	4.4, 1.6, 0.60
ETS exposure					
Yes (*n* = 11)	10.4, 3.5	71.7, 8.0	25.4, 6.0	40.2, 10.4	4.5, 1.9
No (*n* = 28)	9.4, 3.8, 0.47	72.3, 7.5, 0.81	25.7, 7.4, 0.93	40.5, 8.3, 0.91	4.2, 1.8, 0.69
Childcare					
Yes (*n* = 32)	9.4, 3.1	71.4, 7.6	26.0, 7.1	**38.6, 8.3**	4.5, 1.7
No (*n* = 7)	11.1, 5.7, 0.46	75.3, 6.7, 0.22	23.9, 6.0, 0.48	**49.0, 5.1, 0.003**	3.3, 2.2, 0.10
History of URTI					
Yes (*n* = 27)	9.8, 3.9	73.2, 6.6	26.1, 7.2	42.2, 7.5	4.3, 1.9
No (*n* = 12)	9.5, 3.4, 0.84	69.8, 9.1, 0.20	24.4, 6.3, 0.48	36.4, 10.4, 0.06	4.2, 1.8, 0.90

ETS = environmental tobacco smoke; *M* = mean; OM = otitis media; *SD* = standard deviation; T_C = cytotoxic T lymphocyte; T_H = T helper lymphocyte; T_{reg} = regulatory T lymphocyte; URTI = upper respiratory tract infection. Values in bold indicate significance.

4.3 Discussion

4.3.1 Demographic and environmental factors contributing to COM

Although considerable research has been performed on the identification of risk factors for OM, there are many inconsistencies in the findings, due largely to the different research methods used, varying sample sizes, and the diverse environmental factors relevant to different countries. Due to the inconclusive evidence available, it is necessary to compare the results herein carefully and appropriately with similar studies, at least in terms of population age inclusions, and if possible samples sizes and countries with similar cultural and societal norms.

The male to female ratio of children with OM in this study was 1.5:1, which is similar to the OM gender balance reported in Nigeria, America, India, the Netherlands, and Western Australia (Zielhuis et al. 1989; Lamphear et al. 1997; Jacoby et al. 2008; Lasisi et al. 2008; Sophia et al. 2010). In regions such as Sicily though, females are at a higher risk of developing OM compared to males in school-aged children (Martines et al. 2011). The large Nigerian study reported that the mean age for children with OM was 4 years 11 months, older than the average age reported in this study. Whereas the Dutch researchers reported the incidence of OME peaked at 3 years 3 months, which was slightly younger than the average age observed for COM here (Zielhuis et al. 1989; Lasisi et al. 2008).

The number of people living in the household is often identified as a significant risk factor for OM (Zielhuis et al. 1989; Jacoby et al. 2008; Lasisi et al. 2008); a trend with which these findings are comparable. The strength of the Nigerian study may be due to its inclusion of large families that had greater crowding in the household (>10 versus ≤10 persons per household) (Lasisi et al. 2008). In this study it was difficult to test this factor as the average Rockhampton household includes 2.6 persons, so although the study population here was representative of this trend, it was not representative for overcrowding (Australian Bureau of Statistics 2014b). The family sizes in the Netherlands study were similar to those in this study; yet, their sample size was over tenfold greater than here, thereby likely contributing to the power of the significance they achieved with this finding (Zielhuis et al. 1989).

Being the eldest, middle or youngest born child did not significantly increase or decrease the odds of being COM prone. This is contrary to reviewed literature where it is generally considered that having an older sibling increases the risk of developing OM (Rovers et al.

2004). The findings here also differed from the norm in published literature, in which childcare attendance and a sibling history of OM are considered risk factors for developing OM (Zielhuis et al. 1989; Uhari et al. 1996; Lamphear et al. 1997). Given that these three studies predate the pneumococcal vaccine era, and that some variance exists in sample size, care must be taken in these interpretations, as these differences between the studies may contribute to the discrepancies observed. The observation in this study of no increased risk for developing COM in children who attended childcare was similar though, to the outcomes of the Kalgoorlie-Boulder area study where no increased risk of OM development was identified for non-Aboriginal Australian children who attended childcare (Jacoby et al. 2008). It is important to consider that nearly 85% of this study population attended some form of communal care; therefore, it is recognised that a skewed data set such as this, in a limited sample size, can have a large influence on the observed outcomes.

ETS exposure was also found not to be a risk factor for a child being prone to COM. The published literature is confusing on this, yet overall it does seem to favour the view that ETS exposure does contribute to the risk of developing OM (Zielhuis et al. 1989; Stenstrom et al. 1993; Lamphear et al. 1997; Caylan et al. 2006; Sophia et al. 2010; Martines et al. 2011). A meta-analytic review of the literature concluded that exposure to tobacco smoke is a significant risk factor for developing recurrent OM (rOM) or middle ear effusion (MEE) (Strachan & Cook 1998), both of which are associated with COM. Perhaps the skewed data observed herein, where less than 30% of the study population had exposure to tobacco smoke, may help to explain the outcome of ETS exposure not being a risk factor for a child being COM prone. The introduction of pneumococcal vaccines potentially affecting how children develop OM, and therefore if environmental factors such as ETS exposure continue to create increased risk for OM should also be considered. Of note, this outcome was again comparable to the Kalgoorlie-Boulder area study in Western Australia where ETS exposure was reported not to be a significant risk factor for developing OM in non-Aboriginal, Australian children (40% exposure to tobacco smoke), unlike Aboriginal children (64% exposure to tobacco smoke) who were reported to be at a higher risk for developing OM if exposed to tobacco smoke (Jacoby et al. 2008).

The results of this study were also similar to other study outcomes in not identifying upper respiratory infections as risk factors for OM, although the Sicilian study did find that children with a history of URTI had an increased risk of developing OM (Lasisi et al. 2008; Martines et al. 2011).

Although antibiotic therapy within the previous 6 months was a predictor of COM proneness in a univariate analysis, the multivariate analysis showed no significance for it increasing risk of COM proneness. It is important to note that children who are COM prone suffer prolonged or recurrent infections that often require antibiotic treatments to resolve the infections. The high OR for antibiotic therapy within the last 6 months reported in the univariate analysis needs to be interpreted with caution as antibiotic therapy is a consequence of prolonged infection. Considering this, it is likely that a COM prone child would have increased odds of having antibiotic therapy, therefore the results here for antibiotic therapy within the last 6 months would likely relate to consequence rather than a contributing factor for COM proneness.

4.3.2 Demographic and environmental factors contributing to otopathogen colonisation

When considering demographic factors with otopathogen colonisation in the nasopharynx, the results here agreed mostly with current literature findings. Although this study observed no association of age with *S. pneumoniae*, *M. catarrhalis* and NTHi nasopharyngeal culture, a decline in nasopharyngeal colonisation of these bacteria with increasing age has been reported in several studies globally, indicating that these otopathogens are early colonisers (Vaneechoutte et al. 1990b; Faden et al. 1997; Principi et al. 1999; Bogaert et al. 2004; Gunnarsson & Holm 2009). The youngest child of the household has been identified in this study as an independent determinant of otopathogen colonisation, where there was a significant increased risk for the youngest child among siblings having NTHi nasopharyngeal colonisation present, or reduced colonisation with *S. aureus*. Such an increased risk with NTHi colonisation has also been identified in preschool children in Italy and highlights the risk of otopathogen dissemination to young children when older siblings are present (Principi et al. 1999). This outcome is promising in understanding risk among siblings for NTHi colonisation, although given the limited sample size of this study, the result needs to be confirmed in a much larger study cohort.

As mentioned previously, males are more prone than females for developing OM based on gender balance ratios of children with OM (Zielhuis et al. 1989; Lamphear et al. 1997; Jacoby et al. 2008; Lasisi et al. 2008; Sophia et al. 2010). In relation to this it was found that male children had a significant association with positive nasopharyngeal carriage, with males 10 times more likely to have positive otopathogen nasopharyngeal carriage compared to females, although the male gender was not an independent determinant of

nasopharyngeal colonisation. Similarly in the United States, an increased risk of NTHi colonisation, although not *S. pneumoniae* or *M. catarrhalis*, was reported in male children (Pettigrew et al. 2008). In understanding that nasopharyngeal colonisation increases the risk of OM, gender may be a contributing factor for nasopharyngeal colonisation that predisposes a child to an increased risk of OM, although it would be prudent to confirm this association and potential risk factor in a study of a large samples size (Leach et al. 1994; Faden et al. 1997).

This study also demonstrated that ETS exposure was an independent determinant for the increased risk of nasopharyngeal colonisation with *S. aureus* or *M. catarrhalis*. In a larger study, ETS exposure has also been identified to increase the risk of *S. aureus* nasopharyngeal colonisation in children, however, not *S. pneumoniae*, thereby consistent with the findings here (Bogaert et al. 2004). The indication of ETS exposure as an independent determinant of nasopharyngeal carriage is not conclusive. In a large study of 208 children, those exposed to ETS had significantly increased *S. pneumoniae* nasopharyngeal colonisation compared to children without ETS exposure, however similar to this study no such trend was evident for NTHi (Greenberg et al. 2006). Furthermore, children younger than 7 years of age in Italy were not found to have an increased risk of otopathogen colonisation with exposure to ETS (Principi et al. 1999). As the Italian study predates the introduction of pneumococcal vaccines, these results should be compared with caution, given that the pneumococcal vaccine may affect how a child develops OM, including those factors that may increase otopathogen colonisation. Exposure to ETS is a rather vague factor in terms of how it is defined. For example, exposure could differ in duration of exposure (1 month versus 1 year), intensity of exposure (inside the home versus outside the home), and frequency of exposure (once per week versus every day). Such variability between studies could contribute to the inconsistent conclusions observed. This is demonstrated well by Pereiro et al. (2004) where ETS exposure was found to be a risk factor for increased *Neisseria meningitidis* invasive disease in young children, although this was only evident if more than 60 cigarettes were smoked daily in the home, therefore indicating a dose-response relationship (Pereiro et al. 2004).

All other demographic factors were not identified as having significant relationships with nasopharyngeal otopathogen culture, or to significantly increase or decrease the risk of the colonisation. To rule out the risk of a type II error due to a small sample size, it is necessary to test these relationships between demographic factors and nasopharyngeal colonisation

in a considerably larger study cohort. The lack of relationships here between the demographic factors and nasopharyngeal colonisation were not surprising observations though, considering that throughout the literature the conclusions of the relationships and risks of population and environmental factors with nasopharyngeal colonisation are extremely varied. Much of this variability would likely be due to inconsistencies in the parameters used to define the different variables (Principi et al. 1999; Varon et al. 2000; Bogaert et al. 2004; Regev-Yochay et al. 2004; Gunnarsson & Holm 2009; Dunais et al. 2011; Jacoby et al. 2011).

4.3.3 Demographic and environmental factors contributing to lymphocyte distributions in the adenoids and blood

In relation to the lymphocyte profiles of this study population, decreased levels of B and T_H lymphocytes in the blood of children 4 years or older was observed, compared to children 3 years of age or younger, which is comparable to other published studies (Osugi et al. 1995; Comans-Bitter et al. 1997). This study also observed that increasing age was not associated with changes in B, T, T_C, T_H, or T_{reg} lymphocyte percentages from the adenoid, or T and T_C lymphocytes from the blood. Interestingly, for the first time this study reports that increasing age is associated with an increase in the percentage of T_{reg} lymphocytes in the blood; an observation which provides some insight into the maturation of cellular immunity in children. Although this does not provide direct evidence of an association between age and the functionality of T_{reg} lymphocytes, it does suggest that this regulatory lymphocyte population has a raised profile, and thus may strengthen its presence, and potentially increase its role in systemic immunity as children mature.

Although the functionality of T_{reg} lymphocytes to otopathogens was not investigated here, work has been performed in Bristol, of the United Kingdom, to investigate T_{reg} lymphocyte suppression of T_H lymphocyte *S. pneumoniae*-specific immune responses (Pido-Lopez et al. 2011). It was found that T_{reg} lymphocytes exert immune suppression on *S. pneumoniae* antigen-specific $CD4^+$ lymphocyte responses; however, such suppression was only evident in subjects 17 years of age or older. This may provide evidence for maturing regulatory cellular immunity contributing in part to the decline of *S. pneumoniae*-associated disease throughout adolescence.

Although the evidence available concerning the association of demographic factors with lymphocyte percentages in children is minimal, the results of this study for the most part

are consistent with the current literature. A large cohort study of West African children demonstrated lower percentages of T_C lymphocytes in females compared to males; interestingly, the study was inclusive for infants, in whom this difference was most apparent (Lisse et al. 1997). In children 3 years of age or older, no difference in T_C lymphocyte subsets was evident between males and female, which is in accord with the results here. A study involving a large cohort of Asian children also concluded that no significant changes in B, T, T_H and T_C lymphocyte percentages were evident between males and females (Lee et al. 1996).

Although many studies focus on the effects of active cigarette smoke, or *in utero* cigarette smoke exposure on immune function in children, there are only a handful of investigations that have examined the associations of passive cigarette smoke with lymphocyte proportions in children. A similar study to this one, carried out in Italy, showed that children exposed to cigarette smoke had no differences in their adenoid-derived T_H and T_C lymphocyte percentages. However, the peripheral blood-derived T_H lymphocytes were lower in the cigarette smoke-exposed children, in whom the T_C lymphocytes were higher compared to children who were not exposed to cigarette smoke (Avanzini et al. 2006). The difference in results here concerning the T_H and T_C blood lymphocytes may be explained by how tobacco smoke exposure was defined between the Italian study and this study, and the skewing of this study's population in regards to tobacco smoke exposure (28 children not exposed, 11 exposed and 1 not analysed). The investigation by Avanzini et al. (2006) focused primarily on the effects of tobacco smoke exposure on lymphocyte activity, which therefore placed more stringent classifications for determining a child's exposure to cigarette smoke. These included exposure for an average of at least 2 years, with information collected on the number of cigarettes the parent/s smoked per day (Avanzini et al. 2006). The focus of the study described here was to determine the associations of several risk factors with lymphocyte distributions in COM and non-COM prone children. Hence, this study simply determined cigarette smoke exposure based on the child's household exposure and/or their regular exposure in another location, on at least a weekly basis.

There is one other study that has observed similar results to those reported here, for which no associations were evident with passive cigarette smoke exposure, nor correlations or differences in B, T, T_C or T_H lymphocyte percentages in the blood of adolescents (Vardavas et al. 2010). Only naïve T and T_H lymphocyte subsets showed a positive correlation with

passive exposure to cigarette smoke, and levels of these cells were also increased significantly in adolescents who experienced passive exposure to cigarette smoke. In contrast, the memory lymphocyte counterparts had a negative correlation and were decreased significantly in adolescents who were exposed to passive cigarette smoke compared to adolescents who were not (Vardavas et al. 2010). This suggests that the proportion of lymphocytes may not be affected by exposure to cigarette smoke, but rather it is the activity of the lymphocytes that changes. Further supporting this concept are the apparent changes to interferon-gamma (IFN-γ)-producing T_C lymphocytes, in which active cells from the adenoid are reduced significantly in children exposed to cigarette smoke compared to children who are not (Marseglia et al. 2009). This suggests that exposure to cigarette smoke may reduce a child's pro-inflammatory response to viral infection in the nasopharynx, given that IFN-γ is a potent pro-inflammatory cytokine and T_C lymphocytes provide effective cellular immunity to viral infections (Pandiyan et al. 2007).

Following extensive literature searches for possible associations of birth order, childcare attendance and household crowding with lymphocyte proportions in the adenoid and blood of children, it was concluded that very little is known of these relationships. There is one study that has reported no associations or differences in blood T_H and T_C lymphocytes among children of different birth orders (eldest, middle or youngest born child) (Lisse et al. 1997). The results of this study are mostly in agreement, but an increased percentage of T_H lymphocytes in the blood of the youngest child among siblings was observed. Additionally, no correlations or differences were evident in the B, T and T_{reg} lymphocytes from the blood or adenoids in children of different birth orders. Furthermore, no correlations or differences were observed in the B, T, T_C, T_H or T_{reg} lymphocytes derived from the blood or adenoids when analysed against the number of people living in the household. There was however, an interesting negative association of blood-derived T_H lymphocytes in children who attended some form of communal childcare compared to those who did not attend childcare. This result gives weight to the possibility that factors relating to communal childcare influence a child's cellular immunity, perhaps through dissemination of microbial flora in an intimate, closed environment. In the first instance this result needs to be confirmed in a larger study due to the limited sample size of this study. Furthermore, aspects of functional immunity would need to be measured in relation to communal childcare and interpersonal microbial dissemination in order to establish a clear understanding regarding the observed decrease in T_H lymphocytes.

4.4 Conclusion

This is the first known report to present findings on the relationships among demographic and environmental risk factors with nasopharyngeal colonisation and lymphocyte subsets of the adenoid and blood in COM prone children, aged 2 to 7 years from the east coast of Australia in regional Queensland. In relation to this population, common OM risk factors were identified that increased the odds of a child being prone to COM. Male sex, ETS exposure and the youngest child within siblings significantly increased the odds of a child having various trends in nasopharyngeal otopathogen colonisation, which provided evidence of demographic and environmental factors in this region that indirectly increased the odds of developing OM (Lehmann et al. 2008; Daly et al. 2010). These observations warrant further research in a larger scale study to confirm their significance.

This study found that URTI, childcare attendance, birth order (youngest, middle or eldest child among siblings), the number of children in a household, ETS exposure, and gender were not associated with the proportional changes of B, T, T_C, T_H and T_{reg} lymphocytes in the adenoid. Children that had siblings with a history of OM had significantly increased percentages of T and T_C lymphocytes in their adenoid, while these cellular counterparts were significantly decreased in their blood. This may indicate that prior exposure to OM in the home via siblings, influences local and systemic T lymphocyte proportions, yet the importance of these findings in relation to a child being susceptible to COM remain unclear. Circulating B and T_H lymphocytes were decreased in older children, and the latter population were also decreased in children who attended childcare. Such observations suggest circulating lymphocyte proportional changes with age, while also suggesting that factors associated with a closed, crowded environment may influence cellular immunity, perhaps via microbial dissemination. There were significantly less blood-derived T_{reg} lymphocytes in younger children, therefore suggesting that the T_{reg} population increases systemically with age; whether or not these observation support decreased ear disease in adulthood is yet to be elucidated in future functional investigations of T_{reg} lymphocyte suppressive activity and nasopharyngeal carriage with OM.

5 TRENDS IN THE NASOPHARYNGEAL MICROBIOLOGY EVALUATED WITH CLINICAL FACTORS OF CHRONIC OTITIS MEDIA IN CHILDREN FROM RURAL AUSTRALIA

5.1 Introduction

OM is a polymicrobial disease that is caused by both respiratory viruses and bacteria, and which occurs in single or co-infection states (Heikkinen et al. 1999; Massa et al. 2009). It is often a complication of URTI, where AOM episodes occur frequently, leading to COM (Revai et al. 2008). During times of dense nasopharyngeal colonisation, or compromised integrity of the nasopharyngeal mucosa, such as with the presence of a respiratory viral infection, the natural microflora of the nasopharynx ascends the Eustachian tube towards the middle ear (Long et al. 1983; Faden et al. 1990; Radzikowski et al. 2011). In response to microbes entering a sterile site, a strong inflammatory response develops and white blood cells and inflammatory mediators enter the middle ear cavity causing redness, pain and swelling (Sato et al. 1999). The complexity of the disease pathogenesis occurs with the microbial interactions, and microbe-host interactions, leading to the onset of the disease, increased severity of the infections and contributing to chronicity within the disease (Jacoby et al. 2007; Pettigrew et al. 2011; Kaya et al. 2013). This is of course compounded further by host, environmental and microbial factors including but not limited to a person's young age, Eustachian tube dysfunction, the ensuing (or lack thereof) inflammatory response, overcrowding causing rapid dissemination of microbes and dense nasopharyngeal colonisation, and early onset of colonisation (Bluestone & Doyle 1988; Faden et al. 1997; Jacoby et al. 2011). Therefore, it is clear that nasopharyngeal bacteria are a fundamental contributor to causing OM. It is less clear how the inter-bacterial relationships affect OM aetiology.

It is well documented that *S. pneumoniae* is frequently cultured with *M. catarrhalis* or NTHi, and NTHi is frequently cultured with *M. catarrhalis* also, in the nasopharynx of children regardless if OM is present or not (Jacoby et al. 2007; Pettigrew et al. 2008; Casey et al. 2010; Wiertsema et al. 2011). One study investigating nasopharyngeal co-colonisation in rAOM demonstrated that co-colonisation of *S. pneumoniae* with NTHi was increased significantly in children with rAOM compared to healthy children (Wiertsema et al. 2011), suggesting that bacterial synergy is associated with rAOM. Yet a similar study has reported

no significant difference in co-colonisation between groups (Casey et al. 2010). Negative relationships are also documented in published literature where *S. aureus* rarely co-colonisers with *S. pneumoniae* or NTHi in the nasopharynx of children (Regev-Yochay et al. 2004; Zemlickova et al. 2006; Jacoby et al. 2007; Pettigrew et al. 2008). Of note, where *S. pneumoniae* and *S. aureus*, and NTHi and *S. aureus* share an antagonistic relationship in their nasopharyngeal colonisation, human immunodeficiency virus (HIV) infected children have higher carriage rates of *S. pneumoniae* and NTHi. They also exhibit *S. pneumoniae*-*S. aureus* or *S. aureus*-NTHi dual colonisation, thereby suggesting that the host's immunity plays a role in such co-colonisation, and that the relationships do no rely on bacterial interactions alone (Madhi et al. 2007).

When considering the bacterial interactions as part of these colonisation phenomena, current research demonstrates that the trends observed may be species-specific, strain-specific and site-specific (Margolis et al. 2010). A neonatal rodent model established nasopharyngeal colonisation with *S. pneumoniae*, *M. catarrhalis* and *H. influenzae*. Where colonisation was established with *S. pneumoniae* or *S. aureus*, and *H. influenzae* was introduced into the nasopharynx, the dual colonisation density increased, and *H. influenzae* showed an approximate 20% density increase compared to when colonised singularly. This trend was evident in 6 hour broth dual-cultures also (Margolis et al. 2010). When the dual colonisation model was performed in the reverse order, where *H. influenzae* colonisation was pre-established, the introduction of *S. pneumoniae* and *S. aureus* actually demonstrated a decreased colonisation density of the two latter species, however this was not significant (Margolis et al. 2010). Advantageous bacterial interactions have also been demonstrated with *M. catarrhalis* and NTHi dual colonisation *in-vitro*. A possible mechanism of *M. catarrhalis*-induced inhibition of complement-mediated immunity and promotion of NTHi survival in dual colonisation has been reported by Tan et al. (2007). It was shown that in the growth phase of *M. catarrhalis*, outer membrane vesicles (OMV) were released, containing its outer membrane proteins (OMP) UspA1 and UspA2. The OMV neutralised C3 of the complement pathway, inhibiting down-stream complement-mediated immunity. Furthermore, with such immune compromise, NTHi survival was enhanced when incubated in normal human serum pre-treated with UspA1/A2 containing OMV, compared to untreated normal human serum (Tan et al. 2007). These OMV have been identified in children with sinusitis, whom had evident *M. catarrhalis* carriage. Therefore, this

mechanism of *M. catarrhalis* supporting NTHi survival may provide evidence for how such bacterial interactions promote dual colonisation (Tan et al. 2007).

In Western Australia, and more recently New Zealand, studies have investigated nasopharyngeal colonisation trends in children with COM or those prone to COM (Leach et al. 1994; Jacoby et al. 2007; Mills et al. 2015). In high risk Australian Aboriginal populations, children with persistent AOM present with colonisation rates as high as 95%, 82% and 71% for *M. catarrhalis*, *S. pneumoniae* and NTHi, respectively (Gibney et al. 2005). Non-Aboriginal Australian children, however, present with much lower carriage rates of 25%, 25% and 11% for the respective otopathogens (Watson et al. 2006). Non-Indigenous Australian children were also reported to have multiple OM episodes, although the episodes were not always persistent and sometimes they occurred without otopathogen nasopharyngeal colonisation present (Leach et al. 1994). These colonisation rates are all relative to the west coast of Australia, yet otopathogen carriage rates in children from the east coast of regional Australia are unknown, as no such investigation has occurred in this region, where COM is a health concern for young Queensland children (Queensland Government 2009). One study has been performed in the Gold Coast; a metropolitan region of Queensland. NTHi was observed as the leading pathogen in nasal swabs, adenoid swabs and middle ear fluid (MEF) cultures, in children undergoing ventilation tube insertion for OM (Ngo et al. 2015).

For children prone to URTI and their associated nasopharyngeal otopathogen colonisation, studies in the United States and Europe report colonisation rates of approximately 40 - 50%, 50 – 65%, and 30% for *S. pneumoniae*, *M. catarrhalis*, and NTHi, respectively (Varon et al. 2000; Pettigrew et al. 2008; Revai et al. 2008; Usonis et al. 2015). There is no evidence available for comparison of colonisation rates in children prone to URTI from the east coast of Australia, in whom respiratory infections are prevalent. It is important to note that in this study, children with URTI included the diagnosis of tonsillitis and/or OM (information was not gathered on URTI symptoms such as rhinorrhea or sore throat). The definitions of URTI varies, where some are not clearly defined, some are defined by tonsillitis, sinusitis and/or OM, and others are based on symptoms of nasal congestion, rhinorrhea, cough, sore throat, otalgia and/or fever (Varon et al. 2000; Pettigrew et al. 2008; Revai et al. 2008; Usonis et al. 2015). Therefore, the definition of URTI here is comparable to the definitions of URTI reported in literature.

In addressing these unknowns, presented herein is the first study investigating nasopharyngeal colonisation in children of regional Queensland, Australia, who are prone to COM or URTI. The study hypothesis that 'commensal bacteria within the nasopharynx are positively associated with T_{reg} lymphocytes in the adenoids and blood of children with COM, thereby potentially contributing to host-tolerance of nasopharyngeal colonisation and the development of COM', has a strong immunology focus. Although, there are underlying assumptions supporting this hypothesis, in that nasopharyngeal colonisation is present and tolerated in the nasopharynx of children with COM. To confirm these assumptions and provide the microbiological evidence required to support the hypothesis, colonisation trends within the study population are reported here. This includes co-colonisation and inter-bacterial relationships and their associations with a child being prone to COM or URTI. These findings also provide the fundamental microbiological evidence to support the study aims. These include identifying important otopathogens in children prone to COM and URTI in regional Queensland, and determining how bacterial cultures from NPA compare with those from adenoid biopsy cultures to ascertain their value as a screening measure for colonisation within the greater nasopharynx.

5.2 Results

5.2.1 Distribution of otopathogen cultures in the nasopharynx of children

Clinical microbiology was categorised into the following groups; nasopharyngeal colonisation (any otopathogen culture irrespective of culture site), adenoid or nasal colonisation (any otopathogen culture in the adenoid biopsy or NPA, respectively), multiple colonisation (culture positive for two or more otopathogens irrespective of culture site), and *S. pneumoniae*, *M. catarrhalis*, NTHi, *S. aureus*, and other colonisation (species-specific culture irrespective of culture site). A descriptive and frequencies analysis revealed that of all 37 children who had NPA and adenoid biopsies collected, 87% of these were otopathogen culture positive, while 72% and 76% of NPA and adenoid biopsies respectively, were otopathogen culture positive. The distribution of culture positive otopathogens in the nasopharynx of the 37 children is displayed in Table 5.1 where *S. aureus*, *S. pneumoniae*, NTHi and *M. catarrhalis* identified at 38%, 38%, 35% and 24%, respectively. For *M. catarrhalis* the adenoid biopsy and NPA cultures were similar to those in the total nasopharynx. The NTHi NPA cultures were less prominent than those of the total nasopharynx, with only 14% positive culture; although the adenoid biopsy cultures of NTHi were more comparable to the total nasopharynx. On the contrary, for *S. aureus*

positive cultures, those of the NPA were similar to the total nasopharynx, and in comparison to these the adenoid biopsy cultures were decreased. The adenoid and NPA cultures for S. *pneumoniae* were similar, however less than that of the total nasopharynx. Organisms including *S. pyogenes*, Group A Streptococcus and *P. aeruginosa* were all identified at 3% or less of the adenoid biopsy and NPA cultures. *A. otitidis* was culture negative in all 37 children. Of all children in the study, 51% were multiple otopathogen culture positive (two or more otopathogens present in the total nasopharynx).

Table 5.1 Frequencies of otopathogen positive culture in the nasopharynx

Otopathogen positive culture	Adenoid (*n* = 37)	NPA (*n* = 36)	Total Nasopharynx (*n* = 37)
S. pneumoniae	27 (10)	28 (10)	38 (14)
M. catarrhalis	19 (7)	22 (8)	24 (9)
NTHi	27 (10)	14 (5)	35 (13)
S. aureus	27 (10)	36 (13)	38 (14)
S. pyogenes	3 (1)	3 (1)	
Group A Streptococcus	3 (1)	0 (0)	
P. aeruginosa	3 (1)	0 (0)	
A. otitidis	0 (0)	0 (0)	
Overall otopathogen culture	76 (28)	72 (26)	87 (32)
Multiple otopathogen culture			51 (19)

NTHi = non-typeable *H. influenzae*. Values are presented as % (*n*).

5.2.2 Distribution of otopathogen culture in the nasopharynx from children with COM

The frequencies and descriptive analysis for COM prone children and URTI prone children who had cultures from NPA and adenoid biopsies showed that the children in the different groups had a very similar distribution of 83% and 88% otopathogen positive culture, respectively (see Tables 5.2 and 5.3). In the 18 children prone to COM, *S. pneumoniae* was most commonly isolated from the nasopharyngeal sites, with 44% of the children being culture positive, followed by *S. aureus*, *M. catarrhalis* and NTHi, with 39%, 22% and 22% colonisation, respectively. The NPA cultures in the COM prone children varied slightly from this otopathogen culture trend in that *S. aureus* was most frequently isolated from the NPA followed in decreasing order by *S. pneumoniae*, *M. catarrhalis* and NTHi. The adenoid cultures varied from those in the total nasopharynx with NTHi culturing more frequently than *M. catarrhalis* (see Table 5.2). *S. pyogenes* was isolated in less than 6% of all COM prone children, while Group A Streptococcus, *P. aeruginosa* and *A. otitidis* were all culture negative. Of COM prone children 56% were positive for multiple otopathogen

growth in their nasopharynx (see Table 5.2). Figure 5.1 details the main otopathogens isolated from COM and non-COM prone children in both the NPA and adenoid biopsy samples.

Table 5.2 ***Frequencies of otopathogen positive culture in COM prone versus non-COM prone children***

	Adenoid COM		NPA COM		Nasopharynx COM	
Otopathogen positive culture	**COM prone (*n* = 18)**	**Non-COM prone (*n* = 19)**	**COM prone (*n* = 18)**	**Non-COM prone (*n* = 18)**	**COM prone (*n* = 18)**	**Non-COM prone (*n* = 19)**
S. pneumoniae	39 (7)	26 (5)	28 (5)	28 (5)	44 (8)	32 (6)
M. catarrhalis	17 (3)	21 (4)	22 (4)	22 (4)	22 (4)	26 (5)
NTHi	22 (4)	47 (9)	11 (2)	17 (3)	22 (4)	47 (9)
S. aureus	33 (6)	21 (4)	39 (7)	33 (6)	39 (7)	37 (7)
S. pyogenes	6 (1)	0 (0)	6 (1)	0 (0)		
Group A Streptococcus	0 (0)	5 (1)	0 (0)	0 (0)		
P. aeruginosa	0 (0)	5 (1)	0 (0)	0 (0)		
A. otitidis	0 (0)	0 (0)	0 (0)	0 (0)		
Overall Otopathogen	78 (14)	74 (14)	67 (12)	78 (14)	83 (15)	89 (17)
Multiple otopathogen					56 (10)	47 (9)
***S. pneumoniae* + NTHi**					17 (3)	26 (5)
S. pneumoniae* + *M. catarrhalis					22 (4)	11 (2)
***M. catarrhalis* + NTHi**					11 (2)	16 (3)
S. pneumoniae* + NTHi + *M. catarrhalis					11 (2)	11 (2)

COM = chronic otitis media; NTHi = non-typeable *H. influenzae*. Values are presented as % (*n*).

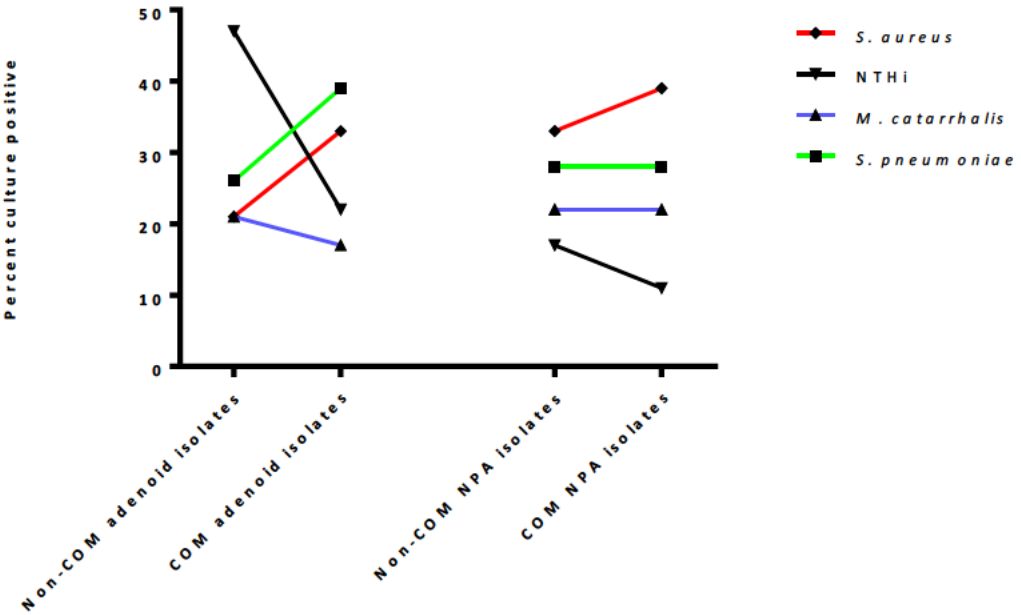


Figure 5.1 Otopathogens among COM and non-COM prone children in NPA and adenoid cultures. Results from chi-squared analysis (no significant differences). Each otopathogen shown as a percentage of culture positive isolates from each site (adenoid or NPA) within each cohort. Non-COM adenoid isolates (*n* = 14), COM adenoid isolates (*n* = 14), non-COM NPA isolates (*n* = 14), COM NPA isolates (*n* = 12).

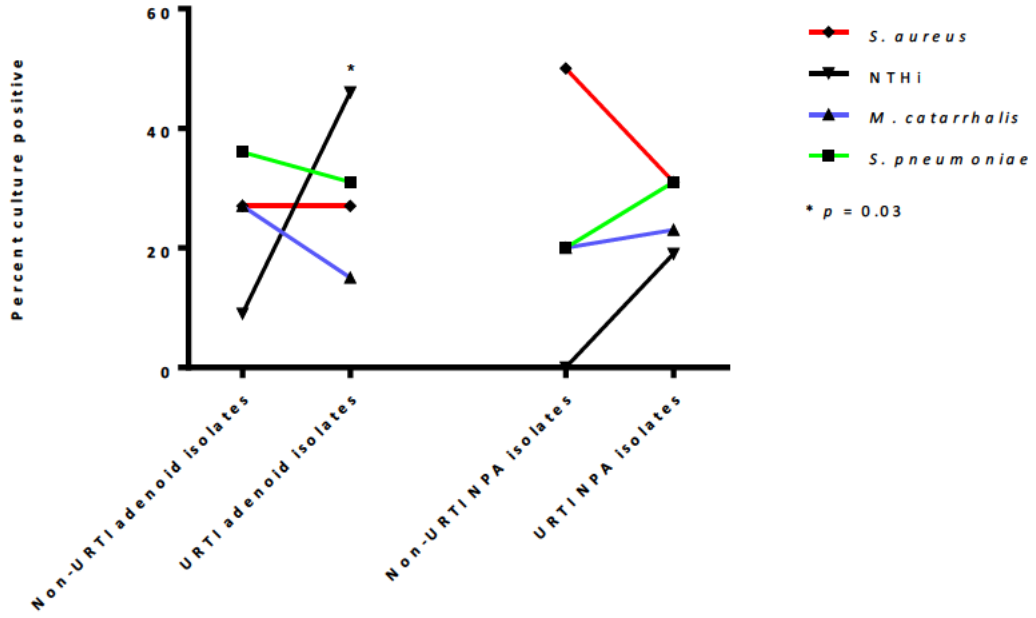
5.2.3 Distribution of otopathogen culture in the nasopharynx from URTI prone children

For the 28 children who were URTI prone (inclusive of tonsillitis and/or OM), 26 had microbiological data from the adenoid biopsy and NPA. Of these, *S. pneumoniae* and *S. aureus* were equally prevalent in the total nasopharynx (collective culture results from the NPA and adenoid biopsies), with 35% of all children testing culture positive for these otopathogens (see Table 5.3). NTHi was isolated most frequently while *M. catarrhalis* was cultured least frequently in the total nasopharynx in 46% and 23% of URTI prone children, respectively. *S. aureus* and *S. pneumoniae* were culture dominant otopathogens in the NPA cultures; however, *M. catarrhalis* carriage in the NPA was similar to that of the total nasopharynx, and NTHi was decreased with only 19% of children prone to URTI having positive NPA cultures. For the adenoid, the otopathogen culture trends resembled closely those of the total nasopharynx, with the main difference observed for *M. catarrhalis* and *S. aureus* cultures declining to 15% and 27%, respectively, compared to the total nasopharynx cultures. *S. pyogenes*, Group A Streptococcus and *P. aeruginosa* were all isolated in less than 4% of URTI prone children, in none of whom *A. otitidis* was isolated. 50% of the URTI prone children cultured positive for more than one otopathogen in the nasopharynx (see Table 5.3). Figure 5.2 shows the main otopathogens isolated from URTI and non-URTI prone children in both the NPA and adenoid biopsy samples.

Table 5.3 Frequencies of otopathogen positive culture in URTI prone & non-URTI prone children

	Adenoid URTI		**NPA URTI**		**Total Nasopharynx URTI**	
Otopathogen positive culture	**URTI prone, *p* value (*n* = 26)**	**Non-URTI prone (*n* = 11)**	**URTI prone (*n* = 26)**	**Non-URTI prone (*n* = 10)**	**URTI prone, *p* value (*n* = 26)**	**Non-URTI prone (*n* = 11)**
S. pneumoniae	31 (8)	36 (4)	31 (8)	20 (2)	35 (9)	45 (5)
M. catarrhalis	15 (4)	27 (3)	23 (6)	20 (2)	23 (6)	27 (3)
NTHi	**46 (12),0.03***	**9 (1)**	19 (5)	0 (0)	**46 (12), 0.03***	**9 (1)**
S. aureus	27 (7)	27 (3)	31 (8)	50 (5)	35 (9)	45 (5)
S. pyogenes	4 (1)	0 (0)	4 (1)	0 (0)		
Group A Streptococcus	4 (1)	0 (0)	0 (0)	0 (0)		
P. aeruginosa	4 (1)	0 (0)	0 (0)	0 (0)		
A. otitidis	0 (0)	0 (0)	0 (0)	0 (0)		
Overall otopathogen	81 (21)	64 (7)	73 (19)	70 (7)	88 (23)	82 (9)
Multiple otopathogen					50 (13)	55 (6)
***S. pneumoniae* + NTHi**					27 (7)	9 (1)
S. pneumoniae* + *M. catarrhalis					15 (4)	18 (2)
***M. catarrhalis* + NTHi**					15 (4)	9 (1)
S. pneumoniae* + NTHi + *M. catarrhalis					12 (3)	9 (1)

NTHi = non-typeable *H. influenzae*; URTI = upper respiratory tract infection. Values are presented as % (*n*). Significant differences are presented in bold. *Fisher's exact test, 1-tailed.



*Fisher's exact test, 1-tailed.

Figure 5.2 Otopathogens among URTI and non-URTI prone children in NPA and adenoid cultures. Results from chi-squared analysis (* indicating significance using Fisher's exact test, 1-tailed where cells had counts of less than 5). Each otopathogen shown as a percentage of culture positive isolates from each site (adenoid or NPA) within each cohort. Non-URTI adenoid isolates (n = 7), URTI adenoid isolates (n = 21), non-URTI NPA isolates (n = 7), URTI NPA isolates (n = 19).

5.2.4 Independent demographic and clinical determinants of colonisation

Previously, a univariate regression analysis was performed to determine if the demographic and clinical factors (see Table 3.1) were possible predictors of colonisation in the nasopharynx. Nasopharyngeal otopathogen colonisation (culture from either NPA or adenoid biopsy), adenoid or NPA otopathogen colonisation, otopathogen culture with two or more species (multiple otopathogen colonisation), *S. pneumoniae*, *M. catarrhalis*, NTHi or *S. aureus* colonisation were the various dependent variables. Any variable with a potentially significant association ($p < 0.10$) was included as a co-variate in a multivariate logistic regression, while also controlling for antibiotics within the last 6 months, with each of the different dependent colonisation outcomes to determine their significance as an independent determinant of colonisation. This showed that ETS exposure had a significant positive effect with *M. catarrhalis* and *S. aureus* colonisation (*M. catarrhalis* OR = 9.04, CI = 1.11 – 73.79, $p = 0.04$; *S. aureus* OR = 44.40, CI = 1.25 – 1583.81, $p = 0.04$), and that the youngest child among siblings had a negative effect with *S. aureus* colonisation, although a positive effect with NTHi colonisation (*S. aureus* OR = 0.01, CI = 0.00 – 0.97, $p = 0.048$; NTHi OR = 17.32, CI = 2.03 – 147.63, $p = 0.009$). All other demographic and clinical factors did not have a significant relationship with the various colonisation outcomes (see Table 4.3b).

5.2.5 Co-colonisation and relationships among bacterial colonisers of children's adenoids

In order to identify relationships among otopathogens of the adenoid, the positive and negative *S. pneumoniae*, *M. catarrhalis*, NTHi and *S. aureus* adenoid cultures were cross-tabulated and analysed by chi-square, reporting significance with Fisher's exact test of independence when cells in the table had frequency values of 5 or less. The proportion of negative *M. catarrhalis* adenoid cultures increased significantly when *S. pneumoniae* culture was also negative in the adenoids ($p = 0.03$, 2-tailed). NTHi negative culture of the adenoids with negative culture of *S. pneumoniae* had a similar trend that was only significant with a 1-tailed test ($p = 0.048$). Negative adenoid culture of *S. aureus* increased significantly when NTHi adenoid culture was present ($p = 0.007$, 2-tailed). In order to determine adenoid species-specific colonisation as a determinant of another species colonisation in the adenoid, univariate logistic regression analysis was used where the effects of *S. pneumoniae*, *M. catarrhalis*, NTHi and *S. aureus* adenoid colonisation were analysed individually, with each species as the dependent variable. Of the adenoid cultures, there were significant positive effects of *M. catarrhalis* and NTHi colonisation on *S. pneumoniae* colonisation, although the negative effect of *S. aureus* colonisation with *S. pneumoniae* colonisation was not significant. When adenoid colonisation of *M. catarrhalis* and NTHi were analysed as co-variates in a binary logistic regression and controlling for antibiotics within the last 6 months, only *M. catarrhalis* was confirmed as an independent determinant of *S. pneumoniae* adenoid colonisation (*M. catarrhalis* OR = 6.90, CI = 1.01 – 47.32, $p = 0.049$). *S. pneumoniae* colonisation was a significant determinant of *M. catarrhalis* colonisation of the adenoid; though when adjusting for the effects of ETS exposure on *M. catarrhalis* colonisation and controlling for antibiotics within the last 6 months, *S. pneumoniae* was confirmed to not be an independent predictor of colonisation. All other colonisers of the adenoid had no significant effect on *M. catarrhalis* colonisation at this site (see Appendix D, Table 9.3). The presence of *S. pneumoniae* had a significant effect on NTHi colonisation in the adenoid. Also, when adjusting for the youngest child among siblings as an independent determinant of NTHi colonisation, and controlling for antibiotics within the last 6 months, *S. pneumoniae* colonisation was confirmed as an independent determinant (OR = 6.89, CI = 1.00 – 47.47, $p = 0.050$). No other colonisers were determinants of NTHi colonisation and all colonisers of the adenoid had no significant effect on *S. aureus* colonisation at this site (see Appendix D, Table 9.3).

5.2.6 Co-colonisation and relationships among bacterial colonisers of the distal nasopharynx in children

In order to identify relationships among otopathogens of the distal nasopharynx, the positive and negative *S. pneumoniae*, *M. catarrhalis*, NTHi and *S. aureus* NPA cultures were cross-tabulated and analysed by chi-square, reporting significance with Fisher's exact test of independence when cells in the table had frequency values of 5 or less. The proportions of negative *M. catarrhalis* or NTHi NPA cultures were significantly higher when *S. pneumoniae* culture was also negative in the NPA (*M. catarrhalis* $p = 0.02$; NTHi $p = 0.02$, 2-tailed). *S. aureus* negative culture of the NPA was also increased significantly, only when positive culture of *S. pneumoniae* was present ($p = 0.005$). In order to determine species-specific colonisation in the distal nasopharynx as a determinant of another species colonisation at the same site, univariate logistic regression analysis was used where the effects of *S. pneumoniae*, *M. catarrhalis*, NTHi and *S. aureus* nasal colonisation were analysed individually, with each species as the dependent variable in the respective model. Nasal colonisation with *M. catarrhalis* or NTHi had a significant positive effect on *S. pneumoniae* colonisation at this site, and when these determinants were analysed in the multivariate logistic regression, controlling for antibiotics within the last 6 months, their independence as determinants of *S. pneumoniae* colonisation in the distal nasopharynx was confirmed (*M. catarrhalis* OR = 15.02, CI = 1.91 – 118.42, $p = 0.01$; NTHi OR = 34.34, CI = 2.42 – 487.53, $p = 0.009$). *S. pneumoniae* colonisation had a significant positive effect with *M. catarrhalis* and NTHi nasal colonisation. However, binary logistic regression analyses adjusting for ETS exposure or for the youngest child among siblings as independent predictors of *M. catarrhalis* and NTHi colonisation, respectively, and controlling for antibiotics within the last 6 months, confirmed *S. pneumoniae* colonisation as an independent determinant of NTHi colonisation, but not of *M. catarrhalis* colonisation in the distal nasopharynx (NTHi OR = 14.16, CI = 1.28 – 156.38, $p = 0.03$). All other associations among the four otopathogens had no significant effects on their colonisation at the distal nasopharynx (see Appendix D, Table 9.3).

5.2.7 Correlations among adenoid and NPA bacterial cultures

To identify how closely associated are the bacterial cultures from the adenoid and distal nasopharynx, the adenoid and NPA positive and negative *S. pneumoniae, M. catarrhalis*, NTHi and *S. aureus* cultures were cross-tabulated and analysed by chi-square, reporting significance with Fisher's exact test of independence when cells in the table had frequency values of 5 or less. The proportions of negative and positive NPA cultures were

significantly higher when adenoid negative or positive culture were present, respectively, for the same bacterial species (see Table 5.4). The exception occurred with NTHi, for which 100% of children with negative adenoid biopsy culture also had negative NPA NTHi culture; however, only 42% of children who had adenoid NTHi positive culture also had NPA NTHi positive culture (see Table 5.4). In order to determine NPA species-specific colonisation as a determinant of the equivalent species colonisation in the adenoid, univariate logistic regression analysis was used where NPA *S. pneumoniae*, *M. catarrhalis*, NTHi or *S. aureus* colonisation were independent variables and the colonisation of the adenoid by these species were dependent variables. This revealed that NPA colonisation with *S. pneumoniae* and *S. aureus* had a significant effect on adenoid colonisation with the equivalent species. In contrast, *M. catarrhalis* and NTHi nasal colonisation did not have an effect on their respective species colonisation on the adenoids (see Table 5.4). In order to confirm *S. pneumoniae* and *S. aureus* NPA cultures as independent predictors for their equivalent species adenoid cultures, binary logistic regression analyses were performed, controlling for antibiotics in the last 6 months and adjusting for the independent determinants of adenoid colonisation: *M. catarrhalis* nasopharyngeal colonisation with *S. pneumoniae* colonisation; and ETS exposure and the youngest child among siblings with *S. aureus* colonisation. This confirmed that children with *S. pneumoniae* NPA positive cultures were almost 40 times more likely to have adenoid *S. pneumoniae* colonisation. Although positive *S. aureus* NPA culture was not an independent predictor of *S. aureus* adenoid colonisation (see Table 5.4).

Table 5.4 Frequencies & associations of adenoid & NPA otopathogen culture

Otopathogen culture	Adenoid	NPA % within Adenoid culture	Fisher's exact test (2-tailed) *p* value	Univariate *n* = 36, OR (95% CI), *p* value	Multivariate *n* = 37, OR (95% CI), *p* value
S. pneumoniae			**0.000**	**30.67 (4.31 – 218.09), 0.001**	**39.30 (3.68 – 419.28), 0.002**
negative	69 (25)	92 (23)			
positive	31 (11)	73 (8)			
M. catarrhalis			**0.000**	ns	ns
negative	83 (30)	93 (28)			
positive	17 (6)	100 (6)			
NTHi			**0.002**	ns	ns
negative	67 (24)	100 (24)			
positive	33 (12)	42 (5)			
S. aureus			**0.000**	**49.50 (4.84 – 505.96), 0.001**	ns
negative	72 (26)	85 (22)			
positive	28 (10)	90 (9)			

CI = confidence interval; NPA = nasopharyngeal aspirate; NTHi = non-typeable *H. influenzae*. Values are presented as % (n).

For the analysis of colonisation associations of NPA otopathogens with other otopathogen species at the adenoids, comparisons were first analysed using the cross-tabulated function and chi-square analyses (Fisher's exact test where cell counts were 5 or less). Direct proportional relationships were evident between *S. pneumoniae* and NTHi NPA and adenoid cultures. NTHi NPA negative cultures were significantly higher when *S. pneumoniae* adenoid culture was also absent, while the same trend was evident for *S. pneumoniae* negative NPA cultures with negative culture of NTHi at the adenoid ($p = 0.02$; $p = 0.007$). Inverse relationships were evident between *S. aureus* and *S. pneumoniae* or NTHi. This was evident with the proportion of *S. aureus* negative NPA cultures significantly higher when *S. pneumoniae* or NTHi adenoid culture were present (*S. pneumoniae* $p = 0.03$; NTHi $p = 0.002$). Negative NPA *S. pneumoniae* cultures were increased with *S. aureus* positive culture at the adenoids, demonstrating the negative association between *S. pneumoniae* and *S. aureus* ($p = 0.04$). The proportions of negative and positive *S. pneumoniae* NPA cultures were significantly higher when *M. catarrhalis* adenoid culture was also negative or positive, respectively ($p = 0.04$).

As co-colonisation trends were equivalent in both culture sites of the nasopharynx, total nasopharyngeal (collective culture results from both nasopharyngeal sites) co-colonisation was analysed between species by chi-square analyses (Fisher's exact test where cell counts were less than 5), and using univariate and binary logistic regression analyses. The results displayed in Table 5.5(a) show the colonisation of otopathogens relative to the co-colonised otopathogen, while the results displayed in Table 5.5(b) demonstrate the nature of the relationships between colonising species. For example, although Fisher's exact test of independence and the univariate logistic regression analyses demonstrate strong, positive associations among *S. pneumoniae, M. catarrhalis* and NTHi, after controlling for the independent determinants of different species-specific colonisation, including ETS exposure and the youngest child among siblings, and controlling for antibiotics within the previous 6 months, *S. pneumoniae* was an independent determinant for NTHi colonisation. However, the presence of *M. catarrhalis* and NTHi in the nasopharynx were not independent predictors for *S. pneumoniae* nasopharyngeal carriage (see Table 5.5b). For *S. pneumoniae* and *S. aureus* colonisation, the relationship was inverse, where children with positive nasopharyngeal culture of either species were 93% less likely to have nasopharyngeal culture of the other species, after adjusting for the youngest child among

siblings and ETS exposure as independent determinants of *S. aureus* colonisation, and controlling for antibiotics within the last 6 months (see Table 5.5b).

Table 5.5(a) Co-colonisation percentages among otopathogen nasopharyngeal culture, (b) Binary logistic regression odds ratios & 95% confidence intervals for co-colonisation predicting otopathogen nasopharyngeal colonisation

a)	***S. pneumoniae* culture within co-colonisation**		***M. catarrhalis* culture within co-colonisation**		***NTHi* culture within co-colonisation**		***S. aureus* culture within co-colonisation**	
Nasopharyngeal culture	negative	positive	negative	positive	negative	positive	negative	positive
S. pneumoniae								
negative	62 (23)		**87 (20)***	13 (3)	**78 (18)***	22 (5)	44 (10)	55 (13)
positive	38 (14)		57 (8)	43 (6)	43 (6)	57 (8)	**93 (13)****	7 (1)
M. catarrhalis								
negative	**71 (20)***	29 (8)	76 (28)		71 (20)	29 (8)	57 (16)	43 (12)
positive	33 (3)	67 (6)	24 (9)		44 (4)	56 (5)	78 (7)	22 (9)
NTHi								
negative	**75 (18)***	25 (6)	83 (20)	17 (4)	65 (24)		42 (10)	58 (14)
positive	39 (5)	61 (8)	62 (8)	38 (5)	35 (13)		100 (13)	**0 (0)*****
S. aureus								
negative	44 (10)	56 (13)	70 (16)	30 (7)	44 (10)	55 (13)	62 (23)	
positive	**93 (13)****	7 (1)	86 (12)	14 (2)	100 (14)	**0 (0)*****	38 (14)	

b)	***S. pneumoniae* nasopharyngeal positive culture**		***M. catarrhalis* nasopharyngeal positive culture**		***NTHi* nasopharyngeal positive culture**		***S. aureus* nasopharyngeal positive culture**	
Risk Factor	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**
***S. pneumoniae* positive nasopharyngeal culture**			**5.00 (1.00 – 25.02), 0.050**	ns	**4.80 (1.13 – 20.46), 0.03**	**7.32 (1.09 – 49.43), 0.04**	**0.06 (0.01 – 0.53), 0.01**	**0.07 (0.01 – 0.70), 0.02**
***M. catarrhalis* positive nasopharyngeal culture**	**5.00 (1.00 – 25.02), 0.050**	ns			ns	ns	ns	ns
NTHi positive nasopharyngeal culture	**4.80 (1.13 – 20.46), 0.03**	ns	ns	ns			ns	ns
***S. aureus* positive nasopharyngeal culture**	**0.06 (0.01 – 0.53), 0.01**	**0.07 (0.01 – 0.84), 0.04**	ns	ns	ns	ns		

CI = confidence interval; NTHi = non-typeable *H. influenzae*; OR = odds ratio. Nasopharyngeal culture is collective for adenoid biopsy and NPA cultures, with results presented for each otopathogen species. Values are presented as % (*n*), **p* = < 0.05, ***p* = < 0.005, ****p* = < 0.000 for Fisher's exact test.

5.2.8 Nasopharyngeal cultures in COM prone children

In order to determine whether otopathogen cultures were significantly related to or differed between COM prone and non-COM prone groups, otopathogen cultures of the nasopharynx and children COM prone and non-COM prone were analysed using the chi-square test and logistic regression. The chi-square analysis revealed no differences in the total nasopharyngeal, adenoid, NPA or different multiple otopathogen cultures in COM prone and non-COM prone children. Furthermore, specific otopathogen positive culture in the general nasopharynx, as well as specific cultures from the adenoid and NPA of *S. pneumoniae*, *M. catarrhalis*, NTHi and *S. aureus* with COM prone children also had no significant relationships or differences between COM prone and non-COM prone children (see Table 5.2). In order to determine if colonisation is a predictor for being COM prone, binary logistic regression analysis was performed, controlling for antibiotic therapy within the previous 6 months. Collective otopathogen nasopharyngeal, adenoid and NPA positive and negative culture, and the different multiple otopathogen culture groups neither increased or decreased significantly the risk of a child being COM prone (see Appendix D, Table 9.4).

5.2.9 Nasopharyngeal cultures in URTI prone children

In order to identify if otopathogen cultures were significantly associated with or differed in children prone to URTI (inclusive of tonsillitis and/or OM) and non-URTI prone children, otopathogen cultures of the nasopharynx from URTI prone and non-URTI prone children were analysed using the chi-square test and logistic regression. The chi-square test (reporting significance with Fisher's exact test of independence for cells with frequency values of 5 or less) demonstrated that the proportion of NTHi positive cultures in the adenoid biopsies and the total nasopharynx were increased significantly in children prone to URTI (adenoid biopsies and total nasopharynx $p = 0.03$, 1-tailed). However, a binary logistic regression analysis adjusted for antibiotic therapy within the previous 6 months indicated that NTHi nasopharyngeal colonisation was not an independent predictor for a child being prone to URTI (OR = 8.57, CI = 0.954 – 77.01, $p = 0.06$).

5.3 Discussion

5.3.1 Otopathogen culture in COM prone children

Many studies have investigated otopathogen colonisation in the nasopharynx and MEE to determine colonisation rates in children with OM. The results herein indicate that in regional Queensland, Australia, 83% of COM prone children have nasopharyngeal

colonisation with at least one otopathogen. *S. pneumoniae* is the dominant otopathogen, with colonisation rates as high as 44%, followed closely by *S. aureus* at 39% and NTHi and *M. catarrhalis* at 22% in children prone to COM, without a current infection. Studies performed in Western Australia and New Zealand of children younger than 3 years of age with rAOM or COM demonstrated similar results in that 79% and 87% of the children from the two areas, respectively, were positive for otopathogens, where both conventional culture techniques and PCR were employed (Wiertsema et al. 2011; Mills et al. 2015). A metropolitan Queensland study that used PCR detection methods also reported similar rates of otopathogen nasopharyngeal positive culture between 78 and 82% in children between 1 and 7 years of age who were OM prone (Ngo et al. 2015). The different colonisation trends between the west and east coasts of Australia and those of New Zealand are interesting. NTHi was the dominant coloniser in children with rAOM or COM, followed by *M. catarrhalis, S. pneumoniae* and *S. aureus*, with carriage rates of 56%, 43%, 41% and 11%, respectively, in Western Australia, and 62%, 57% and 43% respectively in New Zealand for the first three main otopathogens (Wiertsema et al. 2011; Mills et al. 2015). One study performed in the Gold Coast; a metropolitan region of Queensland also identified NTHi as the leading pathogen in nasal swabs, adenoid swabs and MEF cultures, in children undergoing ventilation tube insertion for OM (Ngo et al. 2015). In striking contrast to these Australasian studies, this study's results for a regional area of Queensland showed much lower carriage rates for NTHi and *M. catarrhalis*, and higher carriage for *S. aureus*. *S. pneumoniae* colonisation was similar, while it was also similar to the New Zealand children when the non-COM prone groups were compared (32% in Queensland and 29% in New Zealand) (Mills et al. 2015). Age and colonisation trends must be considered here, as NTHi and *M. catarrhalis* colonisation are known to decline with age, while that of *S. aureus* increases with age. Furthermore, allowing for the fact that the mean age of children in this study was 3.6 years, and included children up to 7 years of age, these could each be a factor contributing to the contrasting colonisation results between the west and east coast Australian children (Vaneechoutte et al. 1990b; Bogaert et al. 2004; Gunnarsson & Holm 2009). In relation to comparing this study with the metropolitan Queensland study, the observed difference for the prominent pathogen in COM prone children may be due to the different detection methods used, where PCR was employed for the metropolitan study rather than conventional culture methods (Ngo et al. 2015).

When considering high risk populations in Australia, in Aboriginal children under 8 years of age with persistent AOM, colonisation rates as high as 95%, 82% and 71% for *M. catarrhalis*, *S. pneumoniae* and NTHi, respectively, have been reported (Gibney et al. 2005). Australian Aboriginal children in the first 2 years of life have average carriage rates of 50%, 49% and 41% for *M. catarrhalis*, *S. pneumoniae* and NTHi, respectively (Watson et al. 2006). Non-Aboriginal Australian children of the same age, however, have much lower carriage rates of 25%, 25% and 11% for the respective otopathogens. Furthermore, the latter have higher carriage rates for *S. aureus* of 61% compared to Australian Aboriginal children with 55% carriage (Watson et al. 2006). Although non-Indigenous Australian children younger than 2 years of age were reported to have multiple OM episodes, each episode was not persistent and sometimes occurred without nasopharyngeal colonisation of the three principle otopathogens (Leach et al. 1994). Collectively, these studies and the complementary findings here indicate that otopathogen carriage in Australian children varies considerably with geography, ethnicity and active infection, and the bacterial aetiology of OM also varies with geography and ethnicity.

Studies outside Australia also demonstrate a degree of variability in nasopharyngeal colonisation rates in children with OM. Using conventional culture techniques, colonisation rates in the nasopharynx and tonsils of Turkish children with OME were less than 10% for *H. influenzae*, *M. catarrhalis* and *S. pneumoniae* (Aydin et al. 2012). For *A. otitidis*, positive culture was observed only in MEE using multiplex PCR, although in Japan, Harimaya et al. (2006) have detected *A. otitidis* in nasopharyngeal swab cultures (almost 11% of children with OM) via PCR detection methods, indicating that PCR is a more sensitive method of detection for this species in the nasopharynx and perhaps explaining why the negative cultures of *A. otitidis* were observed in this study in which conventional culture methods were employed (Harimaya et al. 2006; Aydin et al. 2012). In infants with OM in the United States, the colonisation rates for *M. catarrhalis*, *S. pneumoniae* and NTHi have been reported at 55%, 38% and 19%, respectively. The higher *M. catarrhalis* carriage in the infants may be due to this bacterium being an early, dominant coloniser during the first four years of life, with colonisation declining thereafter (Ejlertsen et al. 1994; Faden et al. 1997). Given that this study included children up to 7 years of age, this may contribute to the lower *M. catarrhalis* carriage that was observed compared to that in the American study.

The current study has shown that *S. pneumoniae* is the dominant coloniser in COM prone children on the east coast of Australia. A similar trend is evident in young children in France, where *S. pneumoniae* is found to be the most prevalent otopathogen in children with AOM (53.5%), although children without AOM (who had non-specific lower or URTI) had 44% pneumococcal nasopharyngeal culture (Varon et al. 2000). High rates between 58 - 66% were also evident in Israeli children, whom were 3 to 48 months of age who had OM (Eldan et al. 2000). Interestingly, in the Aydin et al. (2012) study *S. pneumoniae* was cultured with multiplex PCR at 41% and 44% in the nasal and tonsil swabs, respectively. These results are very similar to those observed here, in which conventional culture methods on NPA and adenoid biopsies were used, perhaps indicating that the chosen combination of screening and sampling techniques will influence the true representation of nasopharyngeal flora (Aydin et al. 2012).

The lower colonisation rates observed by Aydin et al. (2012) compared to this study's results that were attained using similar conventional culture methods, may be explained by differences in the sampling technique. Considering the physical properties of both the bacteria and the nasopharyngeal mucosa, a NPA will not only collect bacteria through the nasopharynx by an abrasive washing motion, it will also include in the aspirate mucous and mucosal cells to which the bacteria adhere, thus providing the opportunity to collect more bacteria for a better representation of the microflora in the nasopharynx. Furthermore, an adenoid biopsy as opposed to a swab will include bacteria in the sample that may originate from biofilms or are resident in the deep crypts of the tissue, a commonly recognised location for bacterial adherence (Swidsinski et al. 2007). One study investigating NTHi in children with OM demonstrated an extremely high nasopharyngeal colonisation rate of 86% using conventional culture methods, although using samples of adenoid cell suspensions. Children without OM also had a large NTHi nasopharyngeal carriage rate of 57%, indicating that the adenoid cell suspension sample method using conventional culture techniques was very effective at determining NTHi nasopharyngeal colonisation (Kodama et al. 1999). Together, these results indicate that compared with other children with OM on a global perspective, COM-prone children on the east coast of Australia have lower *M. catarrhalis* colonisation, similar or lower *S. pneumoniae* colonisation, and variable NTHi nasopharyngeal colonisation. However, it is recognised that it is difficult to make direct comparisons when considering the different ages of the children across studies, different

states of infection (being infection prone versus having a current infection) and the different culture techniques used.

5.3.2 Otopathogen culture in URTI prone children

When children prone to URTI are considered, this study observed NTHi as the leading otopathogen in nasopharyngeal carriage, as 46% of children with a history of chronic URTI, although not a current infection, were colonised with NTHi. This high colonisation rate correlated moderately with the incidence of children being prone to URTI. It was confirmed though, that it was not an independent predictor of a child's susceptibility to URTI, when controlling for antibiotic exposure within the last 6 months. *S. pneumoniae*, *S. aureus* and *M. catarrhalis* were observed at 35%, 35% and 23% rates of nasopharyngeal colonisation, respectively. In a Texan study that included children younger than 3 years of age who had URTI or were prone to URTI, almost 86% and 87%, respectively, were positive for otopathogens, showing similar results to the 88% otopathogen colonisation rate in URTI prone children of this study (Pettigrew et al. 2008; Revai et al. 2008). The *S. pneumoniae* and NTHi colonisation rates in the Texan children with URTI were both 34%, while URTI prone children had rates of 46% and 32%, respectively, similar to this study's observations. This study's results differed in that NTHi carriage rates were slightly higher, and in comparison to their URTI prone group, the *S. pneumoniae* colonisation observed here was lower (Pettigrew et al. 2008; Revai et al. 2008). In France, children from 3 months to 3 years with URTI had colonisation rates of 50% and 30% for *S. pneumoniae* and NTHi, respectively (Varon et al. 2000). This is somewhat at variance to the results reported herein; geographical location, younger age and children enrolled with an active infection in the French study may contribute to this difference. A large scale study in Lithuania reported the colonisation rate of *S. pneumoniae* in children under 6 years of age with URTI to be 41% (Usonis et al. 2015). Therefore, separate studies in Europe, the United States and Australia have reported nasopharyngeal colonisation rates for both *S. pneumoniae* and NTHi in children with or prone to URTI to be between approximately 30% and 50%, where conventional culture methods were used in all cases (Varon et al. 2000; Revai et al. 2008; Usonis et al. 2015). Here the evidence demonstrates that NTHi is a leading otopathogen in the aetiology of URTI in children of regional Queensland, however given the limited sample size, it is necessary to confirm this observation in a larger study cohort.

The colonisation rate of 23% reported herein for *M. catarrhalis* in URTI prone children is lower than previous observations, as the Texan study reported 69% in children with URTI

or 63% in URTI prone children were colonised, while the French study reported 51% (Varon et al. 2000; Pettigrew et al. 2008; Revai et al. 2008). This difference may be due to a number of factors whereby the studies vary, although an important difference was that both the Texan and French studies included nasopharyngeal swabs during active periods of infection. In contrast, the sampling technique used here included NPA and adenoid biopsies for bacteriology in children without current URTI, as sample collection occurred at the time of adenotonsillectomy (although a history of chronic URTI was a contributing factor for adenotonsillectomy). Ejlertsen et al. (1994) reported *M. catarrhalis* carriage rates of 68% during times of URTI, similar to the Texan study; however, post URTI this colonisation reduced to 36%, comparable to the rates that were observed in this study.

5.3.3 Otopathogen relationships in the nasopharynx

Considering that otopathogens co-exist in the nasopharynx asymptomatically, and that multiple bacteria are recovered frequently from the nasopharynx of children with OM and other URTI, in determining the aetiology of OM it is of importance to recognise the inter-pathogen relationships and whether or not these differ in COM and non-COM prone children (Casey et al. 2010; Wiertsema et al. 2011). For instance, the direct bacterial relationships must be elucidated, and at what sites the relationships exist, and if such relationships differ or not between COM and non-COM prone children. In this study, relationships were evident among otopathogens that were similar in the adenoid and nasal culture sites. At both sites *S. pneumoniae* showed positive correlations with *M. catarrhalis* and NTHi, while in the absence of *S. pneumoniae* culture, the proportions of NTHi and *M. catarrhalis* cultures were also significantly reduced. Both of these trends with *S. pneumoniae* are evident in the literature where *S. pneumoniae* is commonly cultured with *M. catarrhalis* or NTHi (Jacoby et al. 2007; Pettigrew et al. 2008; Casey et al. 2010; Wiertsema et al. 2011). In the Western Australian study that included Aboriginal and non-Aboriginal children, *S. pneumoniae* culture associated positively with *M. catarrhalis* culture, and in Aboriginal children *S. pneumoniae* culture was also correlated positively with NTHi culture. Of interest, NTHi was also correlated positively with *M. catarrhalis* culture (Jacoby et al. 2007). Furthermore, a murine model with established nasopharyngeal colonisation has demonstrated symbiotic relationships among otopathogens, particularly *S. pneumoniae* with *M. catarrhalis* and NTHi, which were also enhanced by antecedent respiratory viral infection (Krishnamurthy et al. 2009). *M. catarrhalis* and NTHi also co-colonise within the one biofilm structure *in-vitro*, effectively forming a polybacterial

biofilm, where *M. catarrhalis* produced beta-lactamase in the biofilm conferring protection from ampicillin to susceptible NTHi (Armbruster et al. 2010). Therefore, the results from this study add to the understanding of mutualism in otopathogen dual colonisation, although mechanisms relating to antecedent viral infection and polybacterial biofilms are yet to be elucidated in children, and if such co-colonisation mechanisms differ in children prone or non-prone to COM.

In comparing co-colonisation trends in children with a history of rAOM and in healthy children (without recurrent URTI history), co-colonisation of *S. pneumoniae* with NTHi or *M. catarrhalis*, and NTHi with *M. catarrhalis* was evident in both groups. *S. pneumoniae* colonising with NTHi was increased significantly however, in the rAOM group compared to the healthy group (Wiertsema et al. 2011). In a Rochester study within the state of New York, United States, although co-colonisation trends were evident in OM prone and non-OM prone children, the trends did not differ between the two groups (Casey et al. 2010). Here, it is also shown that co-colonisation trends do not differ between COM prone and non-COM prone children. There is, however, evidence in murine OM models to suggest that co-colonisation of *S. pneumoniae* and *M. catarrhalis* enhances the severity and duration of OM, thereby potentially leading to COM (Krishnamurthy et al. 2009). Collectively, the differences in co-colonisation trends between COM and non-COM prone children may depend on how the cohorts are determined, as all of the studies mentioned, including this one, differ in terms of the definition of 'otitis-prone' and of the parameters that define the control group (some without a history of recurrent URTI, others with decreased severity of OM, and others with a history of URTI, but not OM). In also understanding that non-COM prone does not necessarily mean a healthy control cohort (as the non-COM prone group had other URTI or AH), perhaps clearer differences in the nasopharyngeal bacteriology would be observed in a study that included COM prone children and children without compromised respiratory systems (without URTI or AH), similar to that performed for rAOM by Wiertsema et al. (2011).

In both sites *S. aureus* showed negative associations with NTHi, where the presence of NTHi colonisation was associated with a significant reduction in the proportion of *S. aureus* positive cultures, and these associations did not differ in COM prone children compared to non-COM prone children. This negative relationship was also evident in the Western Australian study in non-Aboriginal children, yet it was not significant (Jacoby et al. 2007). Negative relationships were also observed with *S. aureus* and *S. pneumoniae*, although this

latter relationship was only significant in the distal nasopharynx, where the presence of *S. pneumoniae* was associated with a significant reduction in the proportion of *S. aureus* positive cultures. This trend in childhood nasopharyngeal colonisation associations has been reported in several studies (Regev-Yochay et al. 2004; Zemlickova et al. 2006; Pettigrew et al. 2008). What is interesting is the question of whether this negative association is related to bacterial interactions or to host factors associated with the individual colonisation trends. A large scale study in the Netherlands following *S. pneumoniae* and *S. aureus* colonisation rates from 1 to 19 years of age demonstrated that *S. pneumoniae* colonisation peaks at 3 years of age before declining, whereas *S. aureus* carriage peaks at 11 years of age before it also gradually declines (Bogaert et al. 2004). Hence, it seems that in the present study age associated with carriage rates of the different species contributes to the negative association. However, if the relationship is due to bacterial interactions and competition, it may also be that when pneumococcal carriage declines with age and is 10% or less by 11 years of age, this facilitates an increase in colonisation by *S. aureus* that peaks by 11 years of age before gradually decreasing through to adulthood (Bogaert et al. 2004). In a dual colonisation model assessing the order of species colonised and co-colonisation outcomes, where *H. influenzae* colonisation was first established, the introduction of a second species such as *S. pneumoniae* or *S. aureus* demonstrated a trend of decreased colonisation density of the two latter species (Margolis et al. 2010). Therefore, bacterial interactions are expected contributors to positive or negative co-colonisation outcomes, and such mechanisms remain unknown in nasopharyngeal carriage in children.

5.3.4 NPA cultures reflecting adenoid cultures

Since OM develops from bacteria migrating from the nasopharynx to the middle ear space, it is of fundamental importance to understand the origin of infection in the nasopharynx, in order to understand the pathogenesis of OM (Long et al. 1983; Faden et al. 1990). From a clinical perspective, it has been questioned whether or not NPA cultures are of use for screening measures to determine greater nasopharyngeal colonisation and for relating this to the risk of OM infection. The first step in addressing this issue is to determine if NPA cultures provide an accurate reflection of the microflora of the greater nasopharynx. In order to achieve this first step, the NPA culture results were compared with the adenoid biopsy culture results in the chi-square test (reporting significance with Fisher's exact test for independence where cells had frequency values of 5 or less) and logistic regression to

determine significant associations and predictive value. This showed that otopathogens including *S. pneumoniae*, *M. catarrhalis*, NTHi and *S. aureus* cultures in the NPA had strong positive correlations with the same species cultured from the adenoid biopsies and that if the specific bacterial species cultured negative or positive in the adenoid biopsies the proportion of the same bacterial species culturing negative or positive in the NPA, respectively, was greater. It is important to note, for children with NTHi adenoid negative culture, 100% also had negative NPA NTHi culture, whereas only 42% had positive NPA NTHi culture when adenoid NTHi positive culture was present. For NTHi, this indicates that the use of NPA cultures for screening of otopathogens in the greater nasopharynx is very effective for detecting negative colonisation, but in more than 50% of cases NPA culture will not detect positive NTHi culture relative to the greater nasopharynx. A recent study showed similar results to these findings in that otopathogen nasopharyngeal swab cultures reflected adenoid biopsy cultures, although some variations were present. In cases where *S. aureus* cultures were positive in the adenoid biopsies, only 70% of these children had NPA cultures that reflected this, while *S. pneumoniae* had a higher frequency of NPA cultures (36%) compared to adenoid biopsies cultures (10%) (Torretta et al. 2011). In order to confirm if species-specific NPA cultures are independent predictors of the equivalent species-specific adenoid cultures, binary logistic regression analyses were performed with adjustments for ETS exposure and the youngest child among siblings as independent predictors of different colonising species; these revealed that only *S. pneumoniae*, but not NTHi, *M. catarrhalis* or *S. aureus* NPA cultures were independent predictors of the equivalent species cultured from the adenoids.

It was further considered if NPA cultures could indicate the risk of a child being COM prone; when adjusting for antibiotic exposure within the previous 6 months using binary logistic regression. The likelihood of a child being COM prone with NPA cultures as an independent predictor was not significant. This finding supports those of Radzikowski et al. (2011) where nasopharyngeal otopathogen cultures were weak predictors for AOM. Others have considered the importance of nasopharyngeal cultures as indicators for focused antibiotic therapy. It was reported that 52% of patients had a change to their course of antibiotic therapy on the basis of nasopharyngeal culture results, and that 74% of patients had clinical benefits occurring from such changes (Marzouk et al. 2012). Collectively, the results by Marzouk et al. (2012) and from this study, provide evidence for the benefit of

utilising NPA cultures as a screening tool for colonisation of the greater nasopharynx, and also as a means of focusing antibiotic therapy for improving patient clinical outcomes.

5.4 Conclusion

In conclusion, the findings presented here represent the first report on nasopharyngeal colonisation from children on the east coast of Australia, in regional Queensland, whom are prone to COM and URTI. Through thorough investigation this study could confirm *S. pneumoniae* as the leading nasopharyngeal coloniser in this population of children 2 to 7 years of age who are prone to COM. Furthermore, NTHi was a significant, dominant nasopharyngeal coloniser of individuals within this population who are prone to URTI. Herein, evidence is provided to support the current observations of *S. pneumoniae* with NTHi or *M. catarrhalis* as common co-colonisers. Through rigorous statistical analysis, the study demonstrated the nature of such relationships, finding that *S. pneumoniae* colonisation was a strong predictor for *M. catarrhalis* and NTHi colonisation, supporting the finding in murine models of *S. pneumoniae* pre-existing colonisation enhancing NTHi in nasopharyngeal co-colonisation (Margolis et al. 2010). This may also reflect synergistic mechanisms among co-colonising bacterial species that are yet to be elucidated in children (Krishnamurthy et al. 2009). *S. pneumoniae* was also a strong predictor for negative *S. aureus* colonisation, and vice versa, supporting other observations of the inverse relationship of these otopathogens in children (Bogaert et al. 2004; Regev-Yochay et al. 2004; Zemlickova et al. 2006; Pettigrew et al. 2008). Collectively, the results herein demonstrate that otopathogen colonisation is tolerated in the nasopharynx of children in regional Queensland, although the trends in dominant and subdominant colonisers vary in comparison to global trends. It is also evident that otopathogen co-colonisation trends are similar to those reported elsewhere. These results confirm assumptions of the tolerance to otopathogen nasopharyngeal colonisation in COM prone children, thereby providing the required evidence to support the study hypothesis.

Although all of the above major findings will help to understand the aetiology of COM and URTI, particularly in non-Aboriginal children of regional Queensland, the correlations and strong predictive abilities found with NPA cultures for adenoid nasopharyngeal culture will be of direct benefit to clinicians in making diagnoses. This is the main reason conventional culture techniques were chosen as the study outcomes were principally interested in live bacterial cultures of the nasopharynx; the study findings needed to be relevant to clinicians, for whom clinical cultures are the preferred method of nasopharyngeal microbiological

diagnosis. PCR techniques could be used to confirm the results of this study, but, as discussed earlier, an accurate reflection of the microbiological flora in the nasopharynx does depend on more than just screening methods. By choosing intensive sampling techniques, including adenoid biopsies and NPA, as opposed to swab techniques, this study has attempted to match a stringent sampling method with a clinically relevant screening tool, thereby enabling these results to be of direct relevance to general practitioners and to ENT specialists in applying evidence-based practices.

6 ADENOID AND PERIPHERAL BLOOD LYMPHOCYTE ASSOCIATIONS WITH CLINICAL FACTORS OF CHRONIC OTITIS MEDIA IN CHILDREN FROM RURAL AUSTRALIA

6.1 Introduction

Through both innate and acquired mechanisms of immunity, the mucosal immune system's most important role is to defend the human body against a plethora of potential invading pathogens, while continuing to maintain the symbiotic relationships between the extensive microbiome and the host (Pasare & Medzhitov 2004). To distinguish between, and respond effectively to pathogens and commensals at the mucosal surface, the immune system deploys pattern recognition receptor (PRR) immune surveillance of the microbial load, specialised antigen-sampling cells, primed antigen-specific responses by cellular and humoral mechanisms, and tight regulation via cellular signalling pathways and T_{reg} lymphocytes (Mowat 2003). What is often intriguing about mucosal sites, specifically in respect to the nasopharynx and OM, is that the natural flora have opportunistic characteristics that enable them to transition from harmless commensals to aggressive otopathogens (Vergison 2008). Of the many bacterial otopathogens involved in OM, *S. pneumoniae*, NTHi, and *M. catarrhalis* are the most prevalent nasopharyngeal commensals that engage in virulence shift (Jacoby et al. 2007). The influences on virulence shift are multifactorial and include microbial interactions (both viral and bacterial), microbial carriage load, compromised mucosal integrity, demographic and environmental factors, and host immunity (Hammerschmidt et al. 2005; Jacoby et al. 2007; Kao et al. 2010). Therefore, it is of importance to investigate the relationships between these factors in order to gain a deeper understanding of how disease occurs.

The level of antigen exposure is considered a strong factor influencing the outcome of effector phenotypes and the T_H lymphocyte response. It has been demonstrated that T_H lymphocytes exposed to high dose, continual exposure to the bee venom allergen phospholipase A (PLA) results in the transition from typical T_H1 or T_H2 phenotype expression of IFN-γ or interleukin (IL)-4, respectively, to IL-10 expression with an immunosuppressive response (Meiler et al. 2008). Considering that nasopharyngeal carriage is often high in children prone to COM (Jacoby et al. 2011), it is speculated that induction of an immunosuppressive T_H phenotype may contribute to host tolerance of dense

nasopharyngeal colonisation by otopathogens (Zhang et al. 2011). In support of this hypothesis, evidence of immunosuppressive T_{reg} lymphocytes associated with bacteria and virus in the nasopharynx and at other mucosal sites, has accumulated over the last decade.

T_{reg} lymphocytes are positively associated with, while T_H17 lymphocytes are negatively associated with *S. pneumoniae* nasopharyngeal colonisation, where down-regulated T_H17, and up-regulated suppressive T_H responses to *S. pneumoniae* was observed in children (Zhang et al. 2011; Palomares et al. 2012). In a mouse model of NTHi-induced COM, it has also been demonstrated that T_{reg} lymphocytes are up-regulated in the middle ear mucosa (MEM) of infected mice, compared to uninfected control mice (Hirano et al. 2015). Furthermore, with depletion of the T_{reg} population, almost 100% NTHi clearance in the MEE was achieved, with a reduction in MEM NTHi culture and immunosuppressive cytokines (Hirano et al. 2015). This COM murine model demonstrates well the potential for T_{reg} lymphocytes to support NTHi nasopharyngeal colonisation and promote chronic ear disease, however such associations and immunosuppressive responses remain unknown for *M. catarrhalis* colonisation, or *S. pneumoniae* in relation to COM.

Neisseria meningitidis-specific T_{reg} lymphocytes have been identified in human tonsils, where the suppression of the T_H1 response to *N. meningitidis* has been observed, although the suppression was only evident in the tonsil, and not the peripheral blood lymphocytes (Davenport et al. 2007). Recently it has been demonstrated in murine and *in vitro* models that such T_{reg} immunosuppression may be initiated via meningococcal surface-specific protein induction of monocyte differentiation into macrophage-like cells that upregulate IL-10 and T_H2/T_{reg} lymphocyte-attracting chemokines, rather than pro-inflammatory cytokines (Wang et al. 2016). This demonstrates a potential mechanism of commensal-induced anti-inflammatory responses via the induction of macrophages which induce immunosuppressive cytokines and chemokines, potentially promoting T_H2 and T_{reg} lymphocytes at the nasopharyngeal mucosa. Murine studies of RSV infection also demonstrate that $CD4^+CD25^+$ lymphocytes with immunosuppressive function influx the lung airways, parenchyma and lymph nodes following infection (Fulton et al. 2010). In patients with gingivitis and peridontitis, T_{reg} lymphocytes have been isolated from their oropharynx. *In vitro* experiments has demonstrated that oral Langerhans cells (oLC) or dendritic cells (DC) activated via *Streptococcus mitis*, *Proprionibacterium acnes* and *Bacteroides fragilis* stimulation of toll-like receptor (TLR) 4, induced such T_{reg} lymphocyte expansion (Nakajima et al. 2005; Kopitar et al. 2006; Allam et al. 2008).

Moreover, in other mucosal sites including the gastrointestinal and urogenital tracts, T_{reg} lymphocytes have been associated with microbial flora. Positive correlations are evident with both asymptomatic and gastritis-associated *H. pylori* colonisation of human gastric biopsy samples (Rad et al. 2006; Jang 2010). Mice colonised with intestinal altered Schaedler flora showed increased T_{reg} lymphocytes, compared to mice without the established colonisation. Furthermore, the T_H17 and T_H1 responses were down-regulated, thereby promoting bacterial intestinal colonisation via reduced inflammatory responses (Geuking et al. 2011). The polysaccharide A (PSA) protein from *Bacteroides fragilis*, a commensal of the human colon, has recently been shown to induce human T_{reg} lymphocyte *in vitro*, promoting anti-inflammatory responses to PSA via IL-10 production and tumor necrosis factor (TNF)-α reduction (Telesford et al. 2015). An *in vitro* human study has also demonstrated intestinal-derived *S. aureus* to induce T_{reg} lymphocytes with suppressive function in cord blood from newborns (Rabe et al. 2014). In the urogenital tract, the persistence of human papillomavirus (HPV) infections has also been correlated with T_{reg} lymphocytes inducing immunosuppression, where an increase in the percentage of circulating T_{reg} lymphocytes was observed in patients with persistent HPV16 infection (Walther et al. 2005; Molling et al. 2007). Collectively, there is a plethora of evidence suggesting that T_{reg} lymphocytes are associated with persistent microbial colonisation at mucosal sites. It is plausible that persistent colonisation of otopathogens in the nasopharynx may be associated with T_{reg} lymphocytes, and that such associations may be contributing to bacterial survival in the nasopharynx, thereby promoting COM.

Within Australia and globally, OM is a prevalent paediatric disease of multifactorial aetiology. Hence, it attracts much research of an interdisciplinary nature with investigations spanning across many scientific and medical fields ranging from social implications to host intrinsic factors (Hoffman et al. 2013; Li et al. 2013). What is understood in terms of host immunity and COM is growing, yet much remains unclear with regard to cellular immunity, particularly the roles of T_{reg} lymphocytes in children with OM and of associated microbial and clinical factors (Murphy et al. 2013). In order to gain a better understanding of these, this study has investigated the relationships between lymphocyte subset profiles, including the T_{reg} subset, and the clinical microbiology profile, *S. pneumoniae*-specific total SIgA, PIgA, and PIgG titres, URTI, and COM in children from regional Queensland on the east coast of Australia. It was hypothesised that commensal bacteria within the nasopharynx are positively associated with T_{reg} lymphocytes in the adenoids and blood of children with

COM, thereby potentially contributing to host-tolerance of nasopharyngeal colonisation and the development of COM. Herein, it is demonstrated that hosts' local and systemic immune lymphocyte profiles, including the T_{reg} lymphocyte subset, may be influenced by otopathogen and specific bacterial colonisation loads in the nasopharynx, and lymphocyte profiles are unchanged within URTI or COM clinical disease.

6.2 Results

6.2.1 Correlations among peripheral blood and adenoid lymphocyte subsets

The relationship between peripheral blood and adenoid lymphocyte subsets was investigated using the non-parametric Spearman's rho correlation coefficient. Preliminary analysis revealed a moderate, negative correlation for $CD19^+$ lymphocytes in the PBMC with $CD3^+$ and $CD3^+CD4^+$ lymphocytes in the adenoid ($CD3^+$ r = -0.40, n = 38, p = 0.01; $CD3^+CD4^+$ r = -0.37, n = 38, p = 0.02). The peripheral blood- and adenoid-derived $FoxP3^+CD25^{hi+}CD127^{lo+}$ (T_{reg}) lymphocytes exhibited a strong, positive correlation (r = 0.62, n = 35, p = 0.000). All other lymphocytes from the PBMC and adenoids either had no correlations evident or the correlations were not significant (see Appendix D, Table 9.5).

6.2.2 Lymphocyte subsets in children with COM

An independent Student *t*-test was conducted to compare the lymphocyte subset distributions in the adenoids and peripheral blood of COM prone and non-COM prone children. There were no significant difference in percentages of $CD19^+$, $CD3^+$, $CD3^+CD8^+$, $CD3^+CD4^+$, $FoxP3^+CD25^{hi+}CD127^{lo+}$ lymphocytes in the adenoids or PBMC for COM prone and control children (see Table 6.1).

Table 6.1 Adenoid lymphocyte distributions

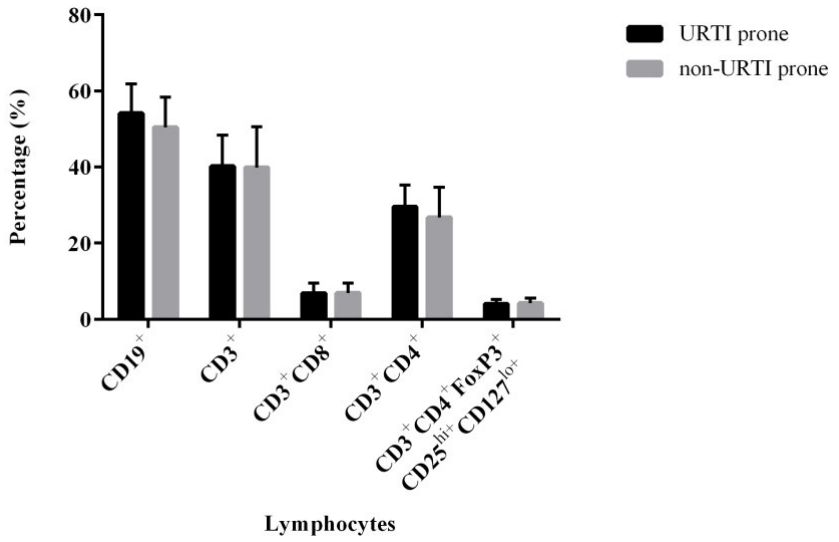
Lymphocyte Subset	COM Prone	Mean	Std. Deviation	Std. Error Mean	*t*	df	Sig. (2-tailed)
Adenoid lymphocytes							
$CD19^+$ B lymphocytes	No	53.1	7.1	1.7	0.103	36	0.92
	Yes	52.8	8.9	1.9			
$CD3^+$ T lymphocytes	No	38.5	9.2	2.2	-1.007	36	0.32
	Yes	41.4	8.8	2.0			
$CD3^+CD8^+$ T_C lymphocytes	No	6.7	2.6	0.6	-0.374	36	0.71
	Yes	7.0	2.6	0.6			
$CD3^+CD4^+$ T_H lymphocytes	No	27.2	5.8	1.4	-1.272	36	0.21
	Yes	29.9	7.0	1.6			
$FoxP3^+CD25^+CD127^{lo+}$ T_{reg} lymphocytes (% T_H lymphocytes)	No	4.4	1.2	0.3	1.457	33	0.15
	Yes	3.8	1.1	0.3			
Peripheral Blood Mononuclear Cells							
$CD19^+$ B lymphocytes	No	10.1	3.8	0.9	0.629	37	0.53
	Yes	9.3	3.7	0.8			
$CD3^+$ T lymphocytes	No	72.0	5.5	1.3	-0.082	37	0.94
	Yes	72.2	9.2	2.1			
$CD3^+CD8^+$ T_C lymphocytes	No	26.6	4.4	1.0	0.924	37	0.36
	Yes	24.6	8.7	1.9			
$CD3^+CD4^+$ T_H lymphocytes	No	39.7	7.1	1.6	-0.527	37	0.60
	Yes	41.2	10.3	2.3			
$FoxP3^+CD25^+CD127^{lo+}$ T_{reg} lymphocytes (% T_H lymphocytes)	No	4.8	2.1	0.5	1.596	37	0.12
	Yes	3.9	1.4	0.3			

COM = chronic otitis media; T_C = cytotoxic T lymphocytes; T_H = T helper lymphocytes. Lymphocyte subsets in COM prone and non-COM prone were analysed by independent student *t*-tests to identify differences in each of the lymphocyte subsets proportions between each cohort (*n* = at least 17 children per group).

6.2.3 Lymphocyte subsets in children with URTI

The relationship between the percentage of PBMC and adenoid lymphocyte subsets with URTI (inclusive of tonsillitis and/or OM) was investigated using the Independent Student *t*-tests. These revealed no significant differences in the blood and adenoid lymphocyte subset percentages in children prone or not prone to URTI (see Figure 6.1).

a)



b)

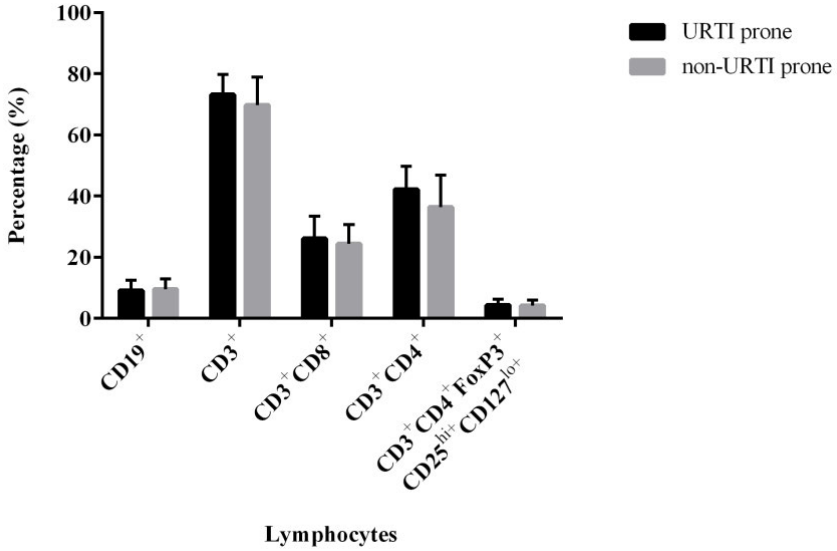


Figure 6.1 Distribution of a) adenoid and b) blood lymphocytes from URTI prone and non-URTI prone children. Results from independent student *t*-tests (no significant differences). $CD19^+$, $CD3^+$, $CD3^+CD8^+$ and $CD3^+CD4^+$ lymphocyte populations shown as a mean percentage with SD of live lymphocytes, and $CD3^+CD4^+FoxP3^+CD25^{hi+}CD127^{lo+}$ lymphocyte population as a mean percentage with SD of $CD3^+CD4^+$ T_H lymphocytes, from each site (adenoid or blood) within each cohort. Non-URTI adenoid (n = 12), URTI adenoid (n = 26), non-URTI blood (n = 12), URTI blood (n = 27).

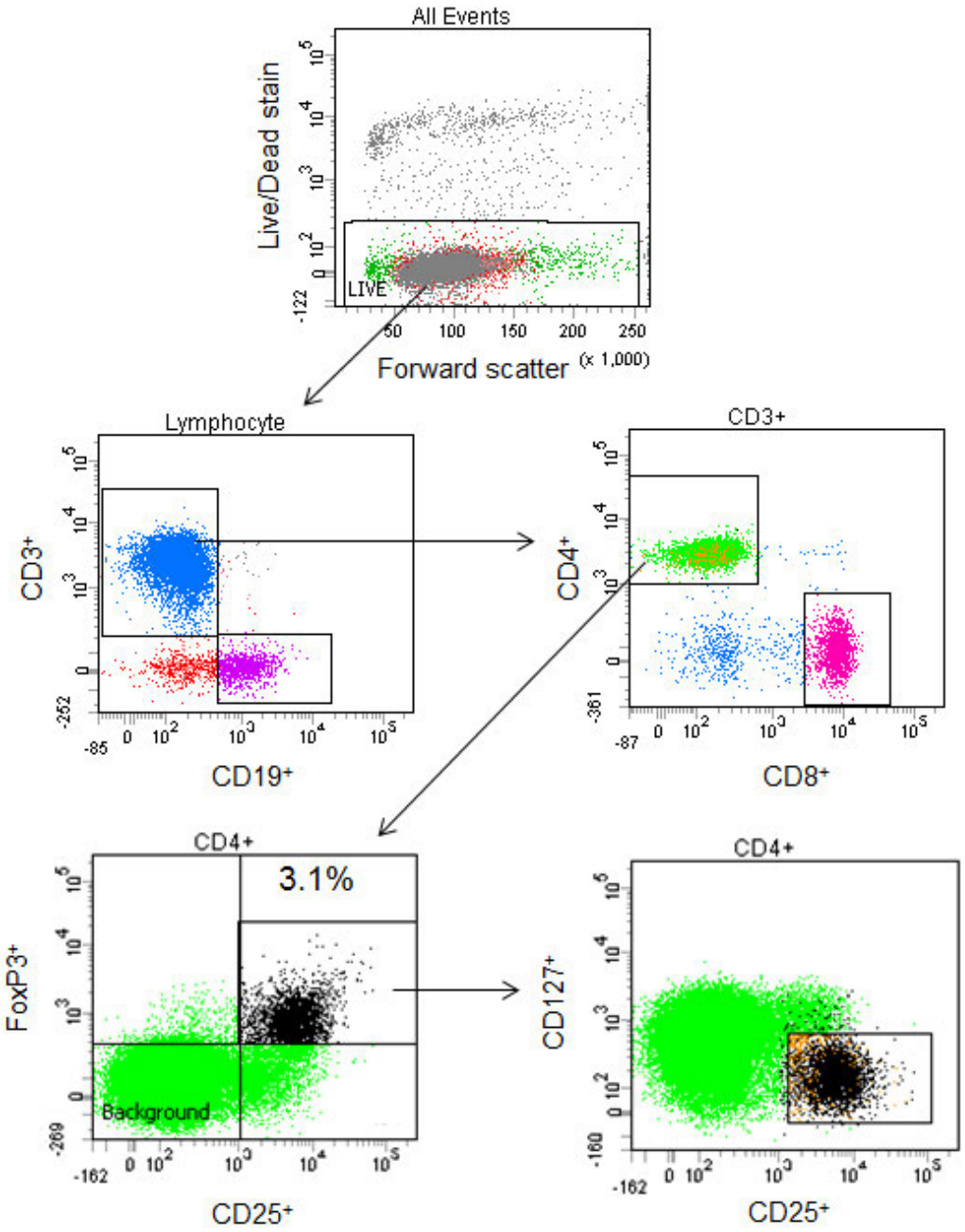
6.2.4 Correlations among lymphocyte subsets and nasopharyngeal colonisation

An independent Student *t*-test was conducted to compare the lymphocyte subset distributions in the adenoids and peripheral blood for nasopharyngeal positive and negative bacterial culture from children. If a culture from a child was positive for one or more bacterial species from their adenoid biopsy or NPA sample, they were considered to have nasopharyngeal positive bacterial culture. For positive bacterial cultures of the nasopharynx, the percentage of PBMC $FoxP3^+CD25^{hi+}CD127^{lo+}$ lymphocytes was greater

compared to nasopharyngeal negative culture (nasopharyngeal positive culture *M* = 4.4%, *SD* = 1.8%; nasopharyngeal negative culture *M* = 3.1%, *SD* = 0.5%, *t* (34) = -3.079, *p* = 0.005) (see Figure 6.2). This difference in the PBMC T_{reg} lymphocytes was also evident in the NPA positive bacterial culture (*M* = 4.6%, *SD* = 1.9%) compared to children with NPA negative culture (*M* = 3.3%, *SD* = 0.6%; *t* (33) = -1.935, *p* = 0.005), and the positive bacterial culture of the adenoid (*M* = 4.5%, *SD* = 1.7%) compared to negative culture of the adenoid (*M* = 3.2%, *SD*; *t* (34) = -2.001 = 1.6%, *p* = 0.050). There were no differences evident in adenoid derived lymphocyte subsets between total nasopharyngeal culture, NPA or adenoid positive and negative bacterial cultures (see Table 6.2).

In analysing associations and differences in peripheral blood- and adenoid-derived lymphocyte subset populations with *S. pneumoniae* positive and negative cultures, no significant relationships or differences were found (see Table 6.2). The percentage of PBMC $CD19^+$ lymphocytes were increased significantly in *M. catarrhalis* culture positive children (*M* = 12.4%, *SD* = 4.0%) compared to *M. catarrhalis* culture negative children (*M* = 8.6%, *SD* = 3.1%; *t* (34) = -2.947, *p* = 0.006). In comparison, adenoid-derived lymphocyte subsets showed no significant differences with *M. catarrhalis* positive culture. In relation to *S. aureus* positive culture, there were no apparent differences in peripheral blood-derived lymphocyte subsets; however, adenoid-derived $CD3^+CD8^+$ lymphocytes were significantly less in *S. aureus* culture positive children (*M* = 5.9%, *SD* = 2.4%) compared to *S. aureus* culture negative children (*M*= 7.7%, *SD* = 2.5%; *t* (33) = 2.188, *p* = 0.04) (see Table 6.2). With NTHi cultures there was an absence of significant differences in the adenoid- or peripheral blood-derived lymphocyte subsets.

a)


All Events
Live/Dead stain
LIVE
Forward scatter (x 1,000)
Lymphocyte
CD3+
CD19+
CD3+
CD4+
CD8+
CD4+
3.1%
FoxP3+
Background
CD25+
CD4+
CD127+
CD25+

b)

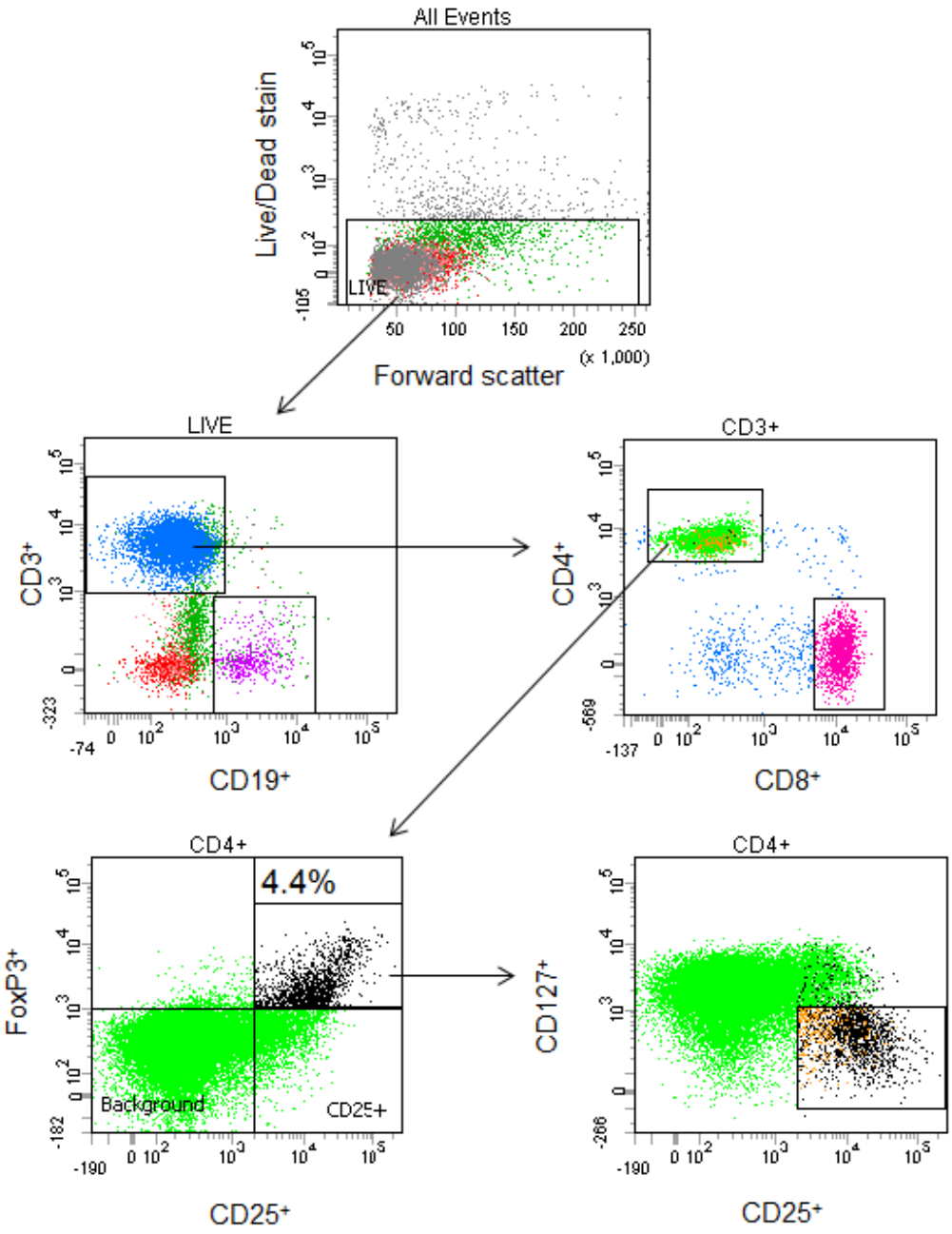


Figure 6.2 T_{reg} lymphocytes in PBMC from a) otopathogen culture negative and b) otopathogen culture positive children. Fluorescent histograms showing gating strategies for $CD3^{+}CD4^{+}FoxP3^{+}CD25^{hi+}CD127^{lo+}$ lymphocyte populations from two representatives of 36 children. Lymphocytes isolated from a single peripheral blood sample from each child were analysed by flow cytometry and differences in lymphocyte populations were assessed in otopathogen culture negative (n= 5) and otopathogen culture positive (n = 26) children using independent student t-tests. Mean percentages of $CD3^{+}CD4^{+}FoxP3^{+}CD25^{hi+}CD127^{lo+}$ lymphocyte populations in otopathogen culture negative and positive children are shown (p = 0.005 where equal variance is not assumed).

Table 6.2 Independent Student t-test for differences in adenoid & blood lymphocyte subsets in nasopharyngeal culture positive or negative children

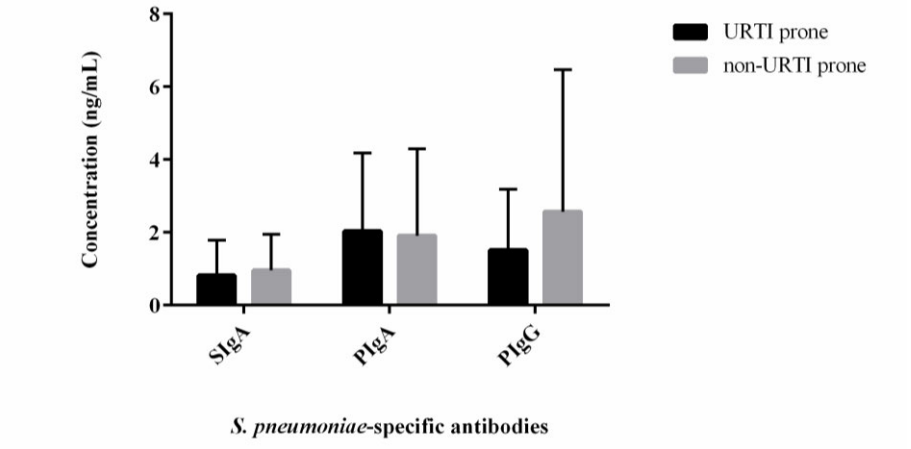
Adenoid-derived lymphocytes						
		B Lymphocytes	**T Lymphocytes**	**T_C lymphocytes**	**T_H lymphocytes**	**T_{reg} lymphocytes**
Nasopharyngeal culture		***M*%, *SD*%, *p* values**	***M*%, *SD*%, *p* value**	***M*%, *SD*%, *p* value**	***M*%, *SD*%, *p* value**	***M*%, *SD*%, *p* value**
Nasopharyngeal otopathogen	-	55.4, 3.7	36.6, 8.7	6.0, 2.8	25.4, 4.5	3.7, 0.5
	+	51.9, 8.3, 0.37	41.1, 9.3, 0.33	7.1, 2.6, 0.40	29.7, 6.4, 0.16	4.1, 1.2, 0.52
Adenoid otopathogen	-	52.8, 6.9	39.1, 8.6	6.9, 2.8	27.8, 5.1	3.9, 1.4
	+	52.3, 8.2, 0.85	40.9, 9.5, 0.61	7.0, 2.6, 0.92	29.6, 6.7, 0.49	4.0, 1.1, 0.84
NPA otopathogen	-	50.8, 10.7	41.7, 12.0	7.0, 2.9	28.6, 9.2	3.6, 0.9
	+	52.8, 6.8, 0.53	40.2, 8.4, 0.69	7.0, 2.6, 0.98	29.4, 5.3, 0.73	4.1, 1.2, 0.25
S. pneumoniae	-	52.2, 9.1	39.8, 10.5	6.4, 2.8	28.8, 7.3	4.0, 1.1
	+	52.9, 4.3, 0.76	41.7, 5.6, 0.57	8.2, 1.8, 0.06	29.8, 3.3, 0.67	4.0, 1.4, 0.98
M. catarrhalis	-	51.2, 8.2	41.0, 10.1	6.9, 2.8	29.5, 7.1	4.0, 1.3
	+	56.0, 5.4, 0.12	38.7, 6.0, 0.52	7.2, 1.9, 0.80	27.9, 2.8, 0.51	3.9, 1.0, 0.82
NTHi	-	53.0, 6.9	39.3, 8.5	6.5, 2.4	28.3, 5.7	4.1, 1.3
	+	50.3, 10.7, 0.40	44.1, 11.1, 0.21	8.5, 2.8, 0.06	31.9, 7.8, 0.16	3.9, 0.7, 0.73
S. aureus	-	52.07, 8.2	41.7, 9.1	**7.7, 2.5**	29.0, 6.3	3.9, 1.1
	+	52.9, 7.6, 0.76	38.5, 9.4, 0.32	**5.9, 2.4, 0.04**	29.3, 6.6, 0.88	4.2, 1.2, 0.50
Blood-derived lymphocytes						
Nasopharyngeal culture		***M*%, *SD*%, *p* values**	***M*%, *SD*%, *p* value**	***M*%, *SD*%, *p* value**	***M*%, *SD*%, *p* value**	***M*%, *SD*%, *p* value**
Nasopharyngeal otopathogen	-	10.0, 2.1	76.2, 4.4	28.7, 6.9	40.7, 9.2	**3.1, 0.5**
	+	9.5, 3.9, 0.75	71.1, 7.7, 0.16	25.0, 7.2, 0.30	40.1, 8.7, 0.90	**4.4, 1.8, 0.005**
Adenoid otopathogen	-	10.5, 4.1	72.8, 5.7	27.8, 8.2	40.6, 8.6	**3.2, 1.5**
	+	9.2, 3.6, 0.38	71.5, 8.0, 0.65	24.8, 6.8, 0.28	40.1, 8.8, 0.88	**4.5, 1.7, 0.050**
NPA otopathogen	-	9.1, 2.4	73.8, 7.8	26.9, 6.3	40.9, 8.7	**3.2, 0.6**
	+	9.5, 4.0, 0.79	70.9, 7.4, 0.33	25.2, 7.6, 0.57	39.6, 8.8, 0.71	**4.6, 1.9, 0.005**
S. pneumoniae	-	9.3, 3.4	70.9, 8.0	24.4, 6.9	38.9, 8.2	4.0, 1.4
	+	10.1, 4.3, 0.55	73.6, 6.2, 0.32	27.8, 7.3, 0.19	42.8, 9.2, 0.20	4.6, 2.3, 0.41
M. catarrhalis	-	**8.6, 3.1**	72.1, 8.2	26.1, 7.4	39.4, 8.0	4.2, 1.6
	+	**12.4, 4.0, 0.006**	71.0, 4.8, 0.73	23.8, 6.2, 0.41	42.4, 10.6, 0.38	4.2, 2.3, 0.95
NTHi	-	9.7, 3.7	70.8, 7.6	24.5, 7.5	39.9, 8.6	3.9, 1.7
	+	8.9, 3.6, 0.58	74.8, 6.7, 0.17	28.7, 5.2, 0.12	41.0, 9.1, 0.76	5.1, 1.9, 0.08
S. aureus	-	10.1, 3.9	73.2, 6.7	27.1, 6.6	41.9, 8.6	4.4, 2.0
	+	8.6, 3.3, 0.22	69.7, 8.3, 0.18	23.0, 7.5, 0.09	37.5, 8.3, 0.14	3.9, 1.2, 0.54

M = mean; NPA = nasopharyngeal aspirate; NTHi = non-typeable *H. influenzae*; *SD* = standard deviation; T_C = cytotoxic T lymphocyte; T_H = T helper lymphocyte. Values in bold indicate significance in Independent student *t*-test and B, T, T_C and T_H lymphocytes presented as a mean percentage of live total lymphocytes, and T_{reg} lymphocytes presented as a mean percentage of live T_H lymphocytes (*n* = at least 36 children).

6.2.5 S. pneumoniae-specific total IgA and IgG titres in children with COM or URTI

The Ig data were tested using frequency histograms to determine the data distribution. The *S. pneumoniae*-specific total SIgA and PIgA or PIgG, respectively data did not follow normal distribution, therefore non-parametric tests were used to compare differences in the titres (Mann-Whitney U-tests) in COM prone and non-COM prone children, and in children with and without URTI (inclusive of tonsillitis and/or OM). Based on these tests the SIgA, PIgA and PIgG titres were not significantly different between COM prone and non-COM prone, or children with and without URTI (see Figure 6.3).

a)



b)

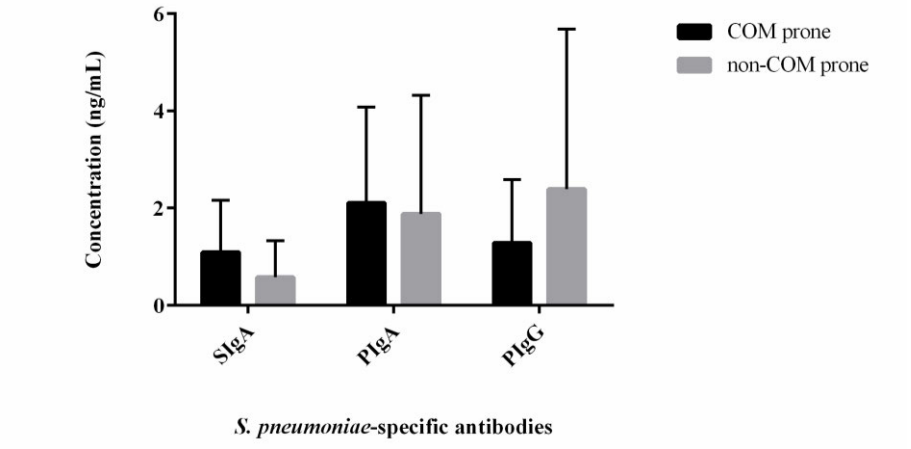
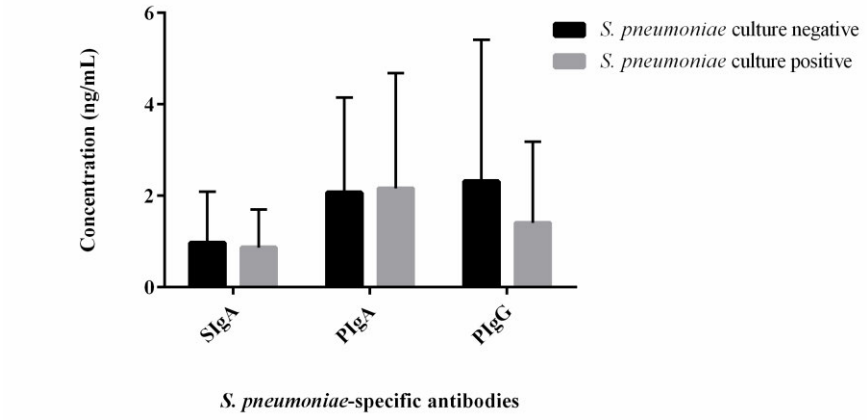


Figure 6.3 *S. pneumoniae*-specific total SIgA, PIgA and PIgG titres in a) COM prone and non-COM prone children, and b) URTI prone and non-URTI prone children. Results from Mann-Whitney U-tests (no significant differences). SIgA, PIgA and PIgG titres shown as a mean concentration (ng/mL) with SD in saliva or plasma samples measured in duplicates, respectively, within each cohort. Non-URTI prone saliva (n = 11), URTI prone saliva (n = 26), non-URTI prone plasma (n = 12), URTI prone plasma (n = 28), non-COM prone saliva (n = 17), COM prone saliva (n = 20), non-COM prone plasma (n = 20) and COM prone plasma (n = 20).

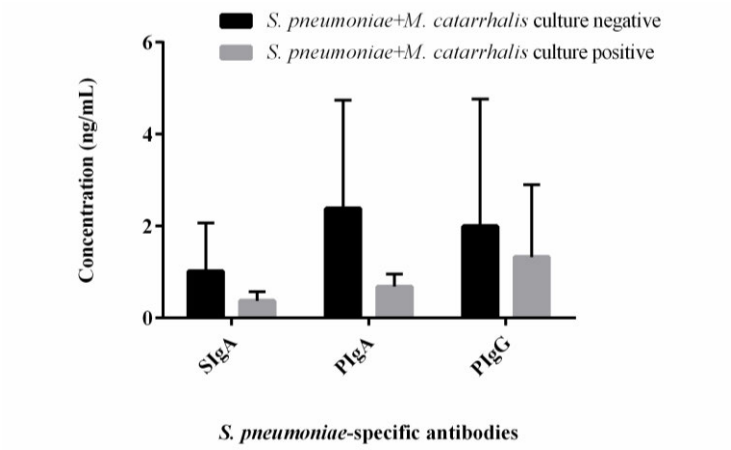
6.2.6 S. pneumoniae-specific total IgA and IgG titres and nasopharyngeal colonisation

Mann-Whitney U-tests were performed in order to determine differences in the *S. pneumoniae*-specific total SIgA, PIgA and PIgG titres with positive and negative pneumococcal-associated culture in children. Included in the comparisons were children with positive or negative nasopharyngeal *S. pneumoniae* culture, dual *S. pneumoniae* + *M. catarrhalis* culture, dual *S. pneumoniae* + NTHi culture, and triple *S. pneumoniae* + *M. catarrhalis* + NTHi culture. The Mann-Whitney U-tests revealed no significant differences in *S. pneumoniae*-specific SIgA, PIgA and PIgG titres with pneumococcal-associated positive or negative carriage (see Figure 6.4).

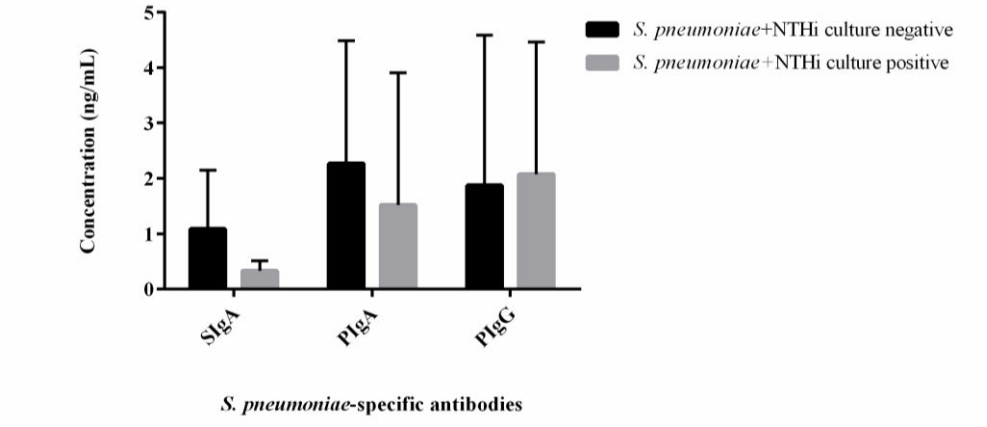
a) S. pneumoniae single culture



b) S. pneumoniae + M. catarrhalis dual culture



c) S. pneumoniae + NTHi dual culture



d) S. pneumoniae + M. catarrhalis + NTHi triple culture

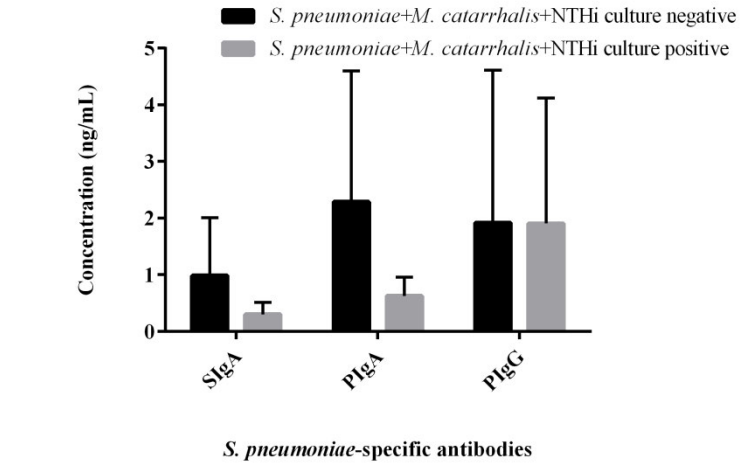


Figure 6.4 *S. pneumoniae*-specific total SIgA, PIgA and PIgG titres in children with positive or negative nasopharyngeal *S. pneumoniae*-associated cultures. Results from Mann-Whitney U-tests (no significant differences). SIgA, PIgA and PIgG titres shown as a mean concentration (ng/mL) with SD in saliva or plasma samples measured in duplicates, respectively, within each cohort. *S. pneumoniae* culture negative saliva (*n* = 20), *S. pneumoniae* culture positive saliva (*n* = 14), *S. pneumoniae* culture negative plasma (*n* = 23), *S. pneumoniae* culture positive plasma (*n* = 14), *S. pneumoniae*+*M. catarrhalis* culture negative saliva (*n* = 28), *S. pneumoniae*+*M. catarrhalis* culture positive saliva (*n* = 6), *S. pneumoniae*+*M. catarrhalis* culture negative plasma (*n* = 31), *S. pneumoniae*+*M. catarrhalis* culture positive plasma (*n* = 6), *S. pneumoniae*+NTHi culture negative saliva (*n* = 26), *S. pneumoniae*+NTHi culture positive saliva (*n* = 8), *S. pneumoniae*+NTHi culture negative plasma (*n* = 29), *S. pneumoniae*+NTHi culture positive plasma (*n* = 8), *S. pneumoniae*+*M. catarrhalis*+NTHi culture negative saliva (*n* = 30), *S. pneumoniae*+*M. catarrhalis*+NTHi culture positive saliva (*n* = 4). *S. pneumoniae*+*M. catarrhalis*+NTHi culture negative plasma (*n* = 33), *S. pneumoniae*+*M. catarrhalis*+NTHi culture positive plasma (*n* = 4).

6.2.7 Correlations among S. pneumoniae-specific total IgA and IgG titres and lymphocyte subsets

The relationship between *S. pneumoniae*-specific total SIgA, PIgA and PIgG, with adenoid or peripheral blood lymphocyte subsets was investigated using the non-parametric Spearman's rho correlation coefficient. A moderate, negative correlation for *S. pneumoniae*-specific SIgA was observed with $CD8^+$ lymphocytes in the PBMC (SIgA *r* =

-0.40, $n = 36$, $p = 0.02$). All other *S. pneumoniae*-specific IgA and IgG titres from the saliva and plasma either had no correlations evident, or the correlations were not significant (see Appendix D, Table 9.6).

6.3 Discussion

Considerable emphasis is placed on local nasopharyngeal immunity when discussing URTI. Mucosal immunity to pathogens is an integrative defence system that is effective through cellular and humoral components which act both locally and systemically. Therefore, this complexity needs to be kept in mind when aiming to understand how the immune system responds to URTI (Kiyono & Fukuyama 2004). The results shared here demonstrate the proportion of humoral and cellular immune lymphocytes and their relationships at systemic and local sites. While B lymphocytes may have a weak systemic presence, concurrently the T and T_H lymphocytes have a stronger local presence in the adenoids. Furthermore, T_{reg} lymphocytes expand proportionally in the nasopharynx and systemically. These findings may indicate in children that local, cellular immunity and systemic, humoral immunity change in concert, thereby emphasizing the importance of understanding both arms of the adaptive immune response when determining its role in different disease states.

In regard to the distribution of local and systemic lymphocyte subsets with URTI, previous studies report no evident differences in the proportion of tonsillar or peripheral blood B, T, T_H or T_C lymphocytes between inflammatory (tonsillitis) and non-inflammatory tonsils (Rosenmann et al. 1998; Bergler et al. 1999). One group has reported that with recurrent tonsillitis, T lymphocytes from the tonsils are increased, compared to children with AH (López-González et al. 1998; Lopez-Gonzalez et al. 1999). This study observed the trend of increased T_H lymphocytes in the adenoid in children with URTI (tonsillitis and/or OM) compared to children without URTI (AH), however the increase was not significant. Furthermore, the results here have expanded on this by finding that no differences or correlations exist between T_{reg} lymphocytes in the blood and adenoids when compared in children with and without URTI.

It has been established previously using flow cytometric techniques that B, T, T_H and T_C lymphocytes in the blood and adenoid do not differ in children with our without AH (Wysocka et al. 2003; Sade et al. 2011; Wojdas et al. 2011). Furthermore, one study has identified T_{reg} lymphocytes in the adenoids of children with AH to be less than 5% of the

T_H lymphocyte subset (Sade et al. 2011). Unfortunately, a comparison here with these studies was not possible due to the low number of participants in this study without AH (*n* = 2). Interestingly, Sade et al. (2011) also investigated the proportion of T_H17 lymphocytes and the T_H17/T_{reg} ratio in the adenoids and concluded that there was an inverse relationship between AH symptom severity and the T_H17/T_{reg} ratio (Sade et al. 2011). In elucidating the dynamic relationship between T_H17 and T_{reg} lymphocytes, and how their functions must be balanced to maintain tissue homeostasis, it may be that an increased T_H17/T_{reg} ratio in the adenoids contributes to reducing AH symptoms via anti-inflammatory mechanisms, thereby leading one to consider the functional certainty of T_{reg} lymphocytes, not just their proportions.

With respect specifically to COM, this study did not observe any differences between the lymphocyte subsets in children with COM compared to children without COM, nor were any associations detected with the lymphocyte subsets and COM. This is consistent with previous studies that reported similar results concerning the relationships of the B, T, T_H and T_C lymphocytes in the adenoids and blood of children with OME (Lagging et al. 1998; Kotowski et al. 2011). Further investigation demonstrated a decrease in the percentage of the activated equivalents of these subsets from the adenoid only (with $CD69^+$ expression) in OME children compared to control children, suggesting that local adaptive immune activation may be depressed in children with COM (Kotowski et al. 2011). It was speculated that T_{reg} lymphocytes would have an increased presence in children with COM, since T_{reg} lymphocytes are thought to contribute to host-tolerance of commensal bacteria and as children with COM have higher carriage loads of otopathogens in their nasopharynx (Faden et al. 1991; Hemlin et al. 1991; Faden et al. 1997; Jang 2010). In contrary to this hypothesis, this study suggests that there are no significant differences in the T_{reg} lymphocyte percentages in the blood or adenoids of children with or without COM. Rather, the results here may indicate that the proportion of T_{reg} lymphocytes differ systemically with varying nasopharyngeal colonisation, as opposed to with disease state.

It is important to be cautious here with the interpretation of this result, considering that the non-COM prone control group was not a 'healthy' reference group. It is not possible under good ethical practice to remove adenoids from healthy children, therefore the non-COM prone control group had to include adenoids collected from children undergoing adenoidectomy for reasons unrelated to OM such as AH and/or tonsillitis, where no history of OM was reported. Although this was the best control available, it was not ideal, as the

children were not 'healthy', therefore the hypothesis may be on target, yet testing it in a clinical setting remains difficult. Perhaps at least circulating T_{reg} lymphocyte proportions could be measured between COM prone and non-COM prone children where it may be ethically acceptable to collect peripheral blood samples from healthy children, yet measuring T_{reg} lymphocytes locally at the nasopharynx from healthy children, to compare with the lymphocyte populations from COM prone children remains challenging.

In the late 1980s the effect of tonsil aerobic bacterial load on the proportions of lymphocyte subsets in the tonsil and in URTI were investigated (Brodsky et al. 1988). The study showed that tonsillar T_H, T_C and B lymphocytes increased in children with tonsillitis compared to control children with normal tonsils. Although no differences in the lymphocyte subsets were observed, a positive correlation of T_H and B lymphocytes with increased bacterial load on the tonsil was reported (Brodsky et al. 1988). While such correlations were not as evident in this study, an increase in adenoid T_H lymphocytes was observed with nasopharyngeal positive culture, albeit insignificant (see Table 6.2). Adenoid T_{reg} lymphocytes increased only slightly with nasopharyngeal positive culture (not significant). The circulating T_{reg} lymphocytes did increase significantly, as reflected by the direct correlations between adenoid and NPA positive culture. With the significance levels set at less than or equal to 0.05 it could be argued that these significant increases in systemic T_{reg} lymphocytes associated with nasopharyngeal culture could occur by chance simply due to five tests in every hundred expected to show a significant relationship where one does not exist (type I error). In Table 6.2 the results from 70 independent student *t*-tests are presented, where five tests (7%) have shown significance, and at least three of these tests have a significance value of less than 0.01. That is, the probability of these results occurring by chance is less than one in every hundred tests. Furthermore, both sites of otopathogen positive cultures were associated with increased systemic T_{reg} lymphocytes, therefore it is reasonable to consider these results are true and not random.

Colonisation in the nasopharynx occurs early in life and is consistently present thereafter, although the bacterial species and associated strains are transient, particularly with heavy antibiotic use and the PCV-13 routines (Faden et al. 1991; Faden et al. 1997; Hare et al. 2013; Casey et al. 2015). Furthermore, lymphocyte expansion and maturation into effector lineages is influenced by antigen exposure and dose (Guiducci et al. 2005; Geuking et al. 2011). In understanding these concepts and acknowledging that the concentration of bacteria affects culture sensitivity, it may be suggested that increasing colonisation levels

in the nasopharynx act to expand systemic T_{reg} lymphocytes, yet the role of these lymphocytes in a child's susceptibility to increased nasopharyngeal colonisation is yet to be determined (Hallander et al. 1993). Furthermore, it is unclear whether the increased proportion of T_{reg} lymphocytes in the blood of children with nasopharyngeal colonisation is a short term response to colonisation, or is inherent, whereby these children tolerate higher colonisation, thereby having increased potential for otopathogen ascension to the middle ear.

Clinically, the association of the proportion of lymphocytes in the blood and adenoid with *S. pneumoniae* nasopharyngeal carriage is largely unknown. Pneumococcal positive and negative culture children have no evident differences in their proportions of T_H lymphocytes in the blood and adenoids (Zhang et al. 2007). Zhang and associates also suspected that T_{reg} lymphocytes promote bacterial colonisation at the nasopharyngeal mucosa. This supposition was vindicated when they found increased T_{reg} lymphocytes from the adenoids in children with positive pneumococcal culture, as compared to negative pneumococcal culture (Zhang et al. 2011). It was unclear if this study ruled out other nasopharyngeal bacteria, so the questions exist: is the increased T_{reg} lymphocytes in the adenoid associated with pneumococcal culture, or with nasopharyngeal culture in general?; when considering overall nasopharyngeal culture, are the proportions of T_{reg} lymphocytes in the adenoid still greater than those in the blood? In a murine model to assess the impact of pneumococcal colonisation on cellular immunity, no changes in B, T, T_H, T_C or T_{reg} lymphocytes were observed in cervical lymph nodes (Richards et al. 2010). While this is similar to what is reported here, a comparison of the results needs to be interpreted with care, due to the species and tissue variations studied. By investigating the lymphocyte subsets of the adenoid and blood in relation to nasopharyngeal *S. pneumoniae* culture in children, the results here suggest that proportional changes may not be significantly influenced by *S. pneumoniae* carriage, supporting the concept that pneumococcal carriage influences adaptive immunity through active pathways only, rather than by polyclonal lymphocyte expansion.

Previously, it has been shown that *M. catarrhalis* induces proliferation of B lymphocytes from the blood in a dose-dependent manner with the MID protein (Wingren et al. 2002). In a study that included children and adults, *M. catarrhalis* and *S. aureus* induced proliferation of mixed tonsillar lymphocytes and isolated B lymphocytes *ex vivo*. Lymphocyte proliferation was reduced with MID deficient *M. catarrhalis* stimulation yet this was

significant only for isolated B lymphocytes (Jendholm et al. 2008). This suggested that MID might have strong mitogenic effects and that T lymphocytes enhance B lymphocyte responses to MID. The observation herein of increased B lymphocytes in the blood strengthens the concept that *M. catarrhalis* might induce a systemic thymus-independent cellular response. Moreover, it should be noted that this is the first report of clinical *M. catarrhalis* carriage being associated with B lymphocyte expansion in children. Together, these results suggest through both experimental and clinical observations that *M. catarrhalis* may induce local and systemic humoral responses that are enhanced by cellular immunity.

The understanding of NTHi and *S. aureus* colonisation effects on lymphocyte profiles are limited. Recently, in NTHi-induced COM in mice it has been demonstrated that T_{reg} lymphocytes are up-regulated in the MEM, compared to uninfected mice, although the T_{reg} levels in the nasal associated lymphoid tissues and blood were not investigated (Hirano et al. 2015). This study has reported that there were no observed differences in lymphocyte subsets in the adenoids and blood of children with NTHi positive culture. The comparisons here need to be considered with care as species, infection states and sample sites differ between the former study and this study. Adenoid T and B lymphocytes in children are known to respond to the P6 antigen of NTHi, so perhaps this otopathogen in a colonisation state, rather than an infection state, induces functional lymphocyte responses that are not reflected in changes to the lymphocyte subset proportions, similar to what is observed with *S. pneumoniae* nasopharyngeal culture (Kodama et al. 1999). Unlike NTHi and *S. pneumoniae*, *S. aureus* nasopharyngeal culture was associated with decreased adenoid T_C lymphocytes in children. It is understood that several *S. aureus* proteins are potent stimulators of T lymphocytes and can also induce anergy of T lymphocytes (Bachert et al. 2002). Therefore, it is possible that the decreased T_C lymphocytes subset in the adenoids of children with *S. aureus* positive culture is a reflection of such induced subset-specific anergy. The associated mechanisms with such an assumption are not known. The results presented here provide the first evidence of the potential association of nasopharyngeal NTHi and *S. aureus* clinical culture with local and systemic lymphocyte subset profiles, and warrant further investigation in a larger study cohort to confirm the associations.

Although there is a considerable focus on cellular immunity within this study, it was of interest to determine if factors of humoral immunity differ with disease states, or nasopharyngeal colonisation, and if they are associated with lymphocyte subsets from the

adenoids and peripheral blood. *S. pneumoniae*-specific antibody titres are well documented in published literature, especially with follow-up investigations since the introduction of the PCV-13 vaccination regime. Previously, in murine models assessing pneumococcal colonisation and its associations with serum and mucosal antibodies, nasopharyngeal colonisation induced an increase of strain-specific pneumococcal surface protein A (PspA) IgG in serum, with the clearance of *S. pneumoniae* colonisation associated with the increase in PspA-specific serum IgG (McCool & Weiser 2004). Although this was observed at the population level, in individual mice there were no associations observed between the *S. pneumoniae*-specific antibody titres and the pneumococcal colonisation, suggesting that the rise in antibody titres was not associated with nasopharyngeal clearance of pneumococcus (McCool & Weiser 2004). When *S. pneumoniae*-specific antibodies were assessed for their associations with *S. pneumoniae* colonisation changes in the children of the present study, no significant correlations were observed, therefore supporting similar trends observed in the study by McCool & Weiser (2004). SIgA and PIgG to pneumococcal proteins have been reported for children with and without *S. pneumoniae* nasopharyngeal colonisation in Bristol, of the United Kingdom (Zhang et al. 2006). Similar to that reported here, no significant changes in SIgA titres were observed in *S. pneumoniae* culture positive children compared to culture negative children. Serum IgG titres, however, were increased in culture negative children, compared to culture positive children, although this significance was pneumococcal protein-specific (Zhang et al. 2006). Therefore, the trends observed here between the Bristol study and the present study are similar, with the Bristol study enrolling considerably more participants into their study, thereby increasing the power of the study, likely contributing to the statistical significance achieved (Zhang et al. 2006). A Dutch study has shown similar results to those presented here, where no differences in *S. pneumoniae*-specific mucosal IgA (detected in MEF) or serum IgG were detected in children with or without *S. pneumoniae* colonisation (Verhaegh et al. 2012).

Several studies have suggested that protection against pneumococcal carriage is antibody-independent, and CD4-dependent, supporting the hypothesis of McCool & Weiser (2004). In a pneumococcal colonisation murine model, salivary IgA and serum IgG were not detected after three weeks of nasopharyngeal colonisation (Malley et al. 2005). Furthermore, initial pneumococcal colonisation protected against secondary colonisation of the same serotypes in antibody- or CD8-deficient mice, yet not in CD4-deficient mice. Immunisation with *S. pneumoniae* WKC in mice reduced pneumococcal colonisation and

elicited elevated levels of IL-17A compared to adjuvant only immunised mice (Lu et al. 2008). Using *in-vitro* assays with splenocytes depleted of $CD4^+$ lymphocytes and stimulated with pneumococcal WKC, IL-17A was significantly reduced to levels comparable in splenocytes from unimmunised mice, however when the $CD4^+$ lymphocytes were replenished in culture, the IL-17A returned to levels similar to those in non-depleted splenocyte cultures. Such findings suggest that protection against nasopharyngeal pneumococcal colonisation is independent of antibody or $CD8^+$ lymphocyte mechanisms, and is CD4-dependent (Malley et al. 2005; Lu et al. 2008). In blood- and adenoid-derived lymphocytes from children, $CD4^+$ lymphocytes proliferated in response to pneumococcal antigen and produced T_H1 cytokines, suggesting that *S. pneumoniae* can elicit cellular responses in children (Zhang et al. 2007). Moreover, children with positive *S. pneumoniae* nasopharyngeal culture, compared to children without such culture, had significantly reduced proliferation and IFN-γ and TNF-α levels associated with the $CD4^+$ PBMC responses to pneumococcal antigen, further indicating that nasopharyngeal colonisation by pneumococcus may be reduced by systemic $CD4^+$ lymphocyte activity (Zhang et al. 2007).

In the present study, in children with dual and triple colonisation associated with *S. pneumoniae*, a trend of decreased IgA antibody was observed either in saliva or plasma samples; whether or not such a decline in mucosal humoral protection translates to an increase in multi-bacterial colonisation is yet to be determined. Research has only begun to question the associations of nasopharyngeal multi-bacterial colonisation and humoral immunity. A recent study has provided a comparison with the results herein. The published work investigated *S. pneumoniae* co-colonisation with *M. catarrhalis* or NTHi and the associated observed changes in humoral responses in children (Xu & Pichichero 2014). It was found that in children with *S. pneumoniae* co-colonisation with *M. catarrhalis* or NTHi, compared to children with sole *S. pneumoniae* colonisation, serum pneumococcal-specific IgA titres were increased. Within the same comparisons, serum pneumococcal-specific IgG showed some increases, however, the statistical significance was dependent on the pneumococcal antigen used to detect the IgG antibody (Xu & Pichichero 2014). The variance observed between this study and the present study in relation to trends in serum antibody levels with pneumococcal co-colonisation may be due to variations across the studies in terms of pneumococcal antigen used to detect antibody levels, the age of the studies participants, and perhaps the cohort size, where Xu & Pichichero (2014) studied

almost five times the number of participants compared to this study, likely contributing to their observed statistical significance (Hotomi 1999; Xu & Pichichero 2014).

The trends shown here demonstrate that *S. pneumoniae*-specific salivary IgA and systemic IgA and IgG may change with specific trends in colonisation, although similar to when considering the children who are COM or URTI prone, no significant changes are observed in their antibody titres, compared to non-COM and non-URTI prone children, respectively. Although statistical significance was not achieved, perhaps due to the limited sample size, reduced PIgG titres were observed in COM prone children, compared to non-COM prone children. Such trends are observed by others with similar studies. Serum IgG tires to five *S. pneumoniae* antigens have shown decreased antibody titres in COM prone compared to non-COM prone children, although not all pneumococcal antigen-specific IgG titres were significantly reduced (Sharma et al. 2011). Anti-pneumococcal IgG_2 in serum has also been measured in COM prone children, showing a significant reduction in anti-pneumococcal IgG_2, compared to titres in healthy children (Hotomi 1999). This difference in the significance observed between the studies may be due to factors of antigen-specific changes, and variance in sample size. In comparing disease states of OM, *S. pneumoniae*-specific IgA and IgG antibodies in MEF and serum, respectively, have been shown to be unchanged in children with rAOM compared to children with COME (Verhaegh et al. 2012).

Presented here are primarily reports on associations of *S. pneumoniae*-specific SIgA, PIgA and PIgG with lymphocytes in the adenoids and peripheral blood in children. Furthermore, it is the first report of a negative correlation of *S. pneumoniae*-specific SIgA with systemic T_C lymphocytes, suggesting that there may be a negative relationship with the levels of circulating T_C lymphocytes and locally produced mucosal antibody to pneumococcal nasopharyngeal colonisation. Comparison studies are limited, although a recent investigation observed positive correlations that reached significance for serotype-specific serum IgG titres with B lymphocytes in children who received the Prevnar 7 vaccine (Moens et al. 2015). The significance of the associations, however, were serotype-dependent, with some associations not reaching statistical significance. This difference in antigen-specific antibody associations with the B lymphocytes, would likely account for the variance observed between this study's results and that of Moens et al. (2015), as different antigen-specific antibody titres were measured.

To gain optimal antibody titres to pneumococcal antigen, the serotypes included in the WCSA preparation were all serotypes included in the PCV-13 vaccine; these being 6B, 14, 18C and 19F (Jefferies et al. 2011). Considering that the majority of the study population received the PCV-13 vaccine, it was expected that high titres would be observed (Jefferies et al. 2011). Antibody titres, however, ranged from below the limit of detection to almost 11ng/ml. These concentrations were sub-optimal and perhaps may be improved if WKC antigen were used, rather than sonicate antigen, particularly in children immunised with the PCV-13 vaccine, as this vaccine recognises both protein components and polysaccharide capsules of the serotypes (Jefferies et al. 2011). It must be considered though, that the titres were measured from samples where no experimentally induced stimulation of antibody production occurred. Therefore, low baseline titres may be expected in samples derived from individuals who may have had previous pneumococcal exposure, either by immunisation with the PCV-13 vaccine, or via past or present pneumococcal carriage. Furthermore, serotype carriage was not assessed, yet it is plausible that these antibody titres could also depend on the positive or negative carriage of the serotypes used to prepare the *S. pneumoniae* WCSA, as it has previously been shown that pneumococcal antibody immunogenicity differs with serotype (Simell et al. 2002). Such increased serotype-specific titres with homologous serotype carriage for 6B, 14 and 19F were evident in children aged between 12 and 18 months, assessing antibody changes with pneumococcal colonisation in a Finnish OM cohort study (Simell et al. 2002).

6.4 Conclusions

This study has provided a clinical investigation of the relationships of adenoid and blood lymphocyte profiles, and *S. pneumoniae*-specific SIgA, PIgA and PIgG titres with COM, URTI and clinical microbiology in children. In considering the pneumoccoal-specific antibody titre observations together with those of others, it may be suggested that the strength of these humoral responses are antigen-dependent and may decrease in COM prone children, compared to non-COM prone children. Within this study, *S. pneumoniae*-specific salivary and plasma IgA and/or IgG titres were not significantly different with changes in nasopharyngeal colonisation or susceptibility to COM or URTI, although some trends were observed similar to those reported by others. Such antibody titres also showed no associations with adenoid- or blood-derived lymphocytes, except for a negative correlation with $CD8^+$ PBMC, with this being the first known report of such associations.

It is clearly evident that changes in lymphocyte subset proportions were observed with microbial changes in the host rather than with clinical disease. This was particularly evident in the observation of elevated levels of circulating T_{reg} and B lymphocytes with increased nasopharyngeal and *M. catarrhalis* colonisation, respectively. These findings are the first to demonstrate increased circulating T_{reg} lymphocytes associated with positive nasopharyngeal otopathogen colonisation; thus, supporting the hypothesis, at least for systemic immunity, that commensal bacteria within the nasopharynx are positively associated with T_{reg} lymphocytes in the adenoids and blood of children with COM, thereby potentially contributing to host-tolerance of nasopharyngeal colonisation and the development of COM. Such findings also demonstrated that changes in the host's nasopharyngeal flora reflected changes in their local and systemic lymphocyte profiles that were bacterial species-specific.

In respect to species-specific positive and negative cultures, with the lymphocyte subset distributions observed here, in conjunction with active lymphocyte changes observed by others, it may be concluded that species-specific nasopharyngeal colonisation influences cellular immunity through functional lymphocyte changes and expansion of specific lymphocyte subsets. These findings warrant further investigation into otopathogen-induced changes to lymphocyte activity, including antigen-specific responses, with the aim to understand how otopathogens affect host immunity and the aetiology of disease.

7 SUMMARY AND FUTURE DIRECTIONS

7.1 Research Overview

The research described in this thesis contributes to the existing knowledge of OM in Australian children, in particular, in the east coast state of Queensland. This study adds to the understanding of risk factors, nasopharyngeal colonisation, cellular immune subset proportions and pneumococcal-specific antibody titres in saliva and plasma samples from children prone to COM. COM is recognised to be an important childhood disease globally and domestically, yet much research is still needed, particularly in areas of prevalence such as the vast state of Queensland (Queensland Government 2009). Aside from the geographical location, what makes this study unique is its focus on investigating relationships among known demographic and clinical risk factors of ear disease, nasopharyngeal colonisation and associated local and systemic cellular immune phenotypes in children. Important risk factors for otopathogen colonisation have been identified, and demographic factors have been recognised as important determinants of specific cellular subset proportions. Furthermore, it is shown that colonisation influences proportional changes in B and T lymphocyte subsets, rather than the disease state of COM. Herein, the major findings of the study are outlined, with conclusions drawn and recommendations made for important future research that builds on these results, considered in the context of complementary findings in the published literature.

This study aimed to improve the understanding of COM in regional Queensland by conducting a local investigation that identified the demographic risk factors for a child being prone to COM, the trends in their nasopharyngeal colonisation, their adenoid and blood cellular immune subset profiles, and their pneumococcal-specific local and systemic IgA and IgG titres. Furthermore, relationships were determined between these factors. This enabled the identification of independent risk factors for colonisation and the associated impact on lymphocyte profiles, thereby improving the understanding of interrelationships between causal factors and immunity relating to a child's susceptibility to COM. These findings provided the foundations to test the study hypothesis that commensal bacteria within the nasopharynx are positively associated with T_{reg} lymphocytes in the adenoids and blood of children with COM, thereby potentially contributing to host-tolerance of nasopharyngeal colonisation and the development of COM.

Results were achieved by means of detailed comparisons between demographic factors and clinical measures of COM prone and non-COM prone children, including the sample population's demography and clinical history, various otopathogen negative and positive cultures, pneumococcal-specific salivary IgA and plasma IgA and IgG titres, and different lymphocyte subset distributions in the blood and adenoids. An important clinical aim was to also identify if NPA cultures reflected adenoid biopsy cultures, and if these were of clinical significance. This was achieved by further detailed comparisons of the different otopathogen positive and negative cultures from the NPA and adenoid biopsy samples. The conclusions from these results are relevant to general practitioners and ENT physicians in developing screening approaches for diagnoses or prophylactic antibiotic therapies.

The study showed that no demographic or clinical factors were significant predictors of a child's increased risk of COM in regional Queensland, however, give the study's limited sample size, a larger study is required to confirm these results. Demographic factors did however, influence nasopharyngeal otopathogen colonisation significantly. The youngest child among siblings was at a significantly higher risk for NTHi colonisation and a reduced risk for *S. aureus* colonisation. This highlights the dynamics of bacterial dissemination among siblings and the risk younger children encounter in their immediate environment. Although male gender was a contributing factor for increased nasopharyngeal colonisation, it was not an independent predictor of this colonisation. As such, this indicates that a male child may develop increased nasopharyngeal colonisation, thereby indirectly increasing their risk of OM. This finding may help to explain the increased ratio of OM in boys that is commonly observed (Zielhuis et al. 1989; Lamphear et al. 1997; Jacoby et al. 2008; Lasisi et al. 2008; Sophia et al. 2010). The risk of *M. catarrhalis* and *S. aureus* nasopharyngeal colonisation in children increased with ETS exposure. This helps to strengthen the theory that cigarette smoke increases nasopharyngeal otopathogen colonisation, thereby indirectly increasing the risk of a child developing OM. The identified demographic and environmental risk factors potentially increasing nasopharyngeal colonisation provides evidence for larger studies to investigate these relationships to better understand nasopharyngeal colonisation in children and its relevance to COM proneness.

Understanding how demographic and clinical factors affect cellular immune phenotype proportions, both locally at the nasopharynx in relevance to COM, and systemically, is important in appreciating how such alterations to a child's immunity may influence the development of OM. The finding of increased age associated with a decline in B and T_H

lymphocytes in the blood strengthens the prevailing view of age-associated cellular immune profiles (Osugi et al. 1995; Comans-Bitter et al. 1997). It was further revealed that increasing age was not associated with changes in percentages of B, T, T_C, T_H, or T_{reg} lymphocytes from the adenoid. In the blood, T and T_C lymphocytes percentages also did not change, although there was an increase in the levels of T_{reg} lymphocytes. Collectively, this indicates proportional changes to lymphocytes with increasing age occur at the systemic level rather than locally in the nasopharynx. Knowledge of the maturation of cellular immune profiles in children is also strengthened, particularly in reference to the regulatory lymphocyte population, which is found to increase systemically as children mature. The T_{reg} lymphocyte changes in the blood associated with increasing age suggest that the systemic T_{reg} population increases with age; whether or not these observation support decreased ear disease in adulthood is yet to be elucidated. This may be addressed in future functional investigations of T_{reg} lymphocyte suppressive activity and nasopharyngeal carriage with OM in a larger cohort of children, adolescents and adults.

In understanding that this study had a limited sample size, observed trends still provide valuable guidance for future larger studies that investigate the true significant value of the demographic and environmental factors associated with lymphocyte proportions. The majority of demographic and clinical factors assessed including ETS exposure, birth order, household crowding, children with siblings having a history of OM, antibiotic or steroid therapy, and URTI (inclusive of tonsillitis and/or OM) showed no associations with the proportions of the blood- or adenoid-derived B, T, T_C, T_H or T_{reg} lymphocytes. These relationships must not be ruled out until they have been confirmed in a larger cohort of children. Childcare attendance was associated with a significant decrease in T_H blood lymphocytes, providing evidence for this relationship to be explored in a larger study. This evidently supports a child's cellular immunity being influenced by factors to which they are exposed to in communal childcare; while it is reasonable to speculate that microbial dissemination in an intimate closed environment may be involved, aspects of functional immunity would need to be measured in relation to communal childcare and microbial dissemination for a clear understanding to be established regarding the decrease in T_H lymphocytes.

Clinical microbiology investigations showed that 83% of children prone to COM in regional Queensland had nasopharyngeal colonisation with at least one otopathogen, with *S. pneumoniae* colonisation at 44%, followed by *S. aureus* at 39%, and NTHi and *M.*

catarrhalis at 22%. Considering these colonisation rates in light of similar studies across Australia and New Zealand, a more comprehensive understanding of otopathogen carriage in Australasian children is achieved. Collectively, the data demonstrate that geography, ethnicity and the state of infection contribute to carriage variations, yet the bacterial aetiology of OM also varies with geography and ethnicity. It is evident that these factors should be considered in clinical approaches to COM and otopathogen colonisation; hence a child's treatment may differ depending on their demography (Leach et al. 1994; Gibney et al. 2005; Watson et al. 2006; Wiertsema et al. 2011; Mills et al. 2015). From a global perspective comparing the otopathogen colonisation rates in children with OM, COM-prone children in regional Queensland have lower *M. catarrhalis* colonisation, similar or lower *S. pneumoniae* colonisation, and variable NTHi nasopharyngeal colonisation. It is acknowledged, however, that making direct comparisons is difficult when study variations exist, including the children's age, state of infection (being infection prone versus having a current infection) and the different microbial culture techniques used.

Understanding the interactions among otopathogens is integral to the study of OM pathogenesis, given its multi-bacterial dynamics. At the adenoid and nasal culture sites *S. pneumoniae* showed positive correlations with *M. catarrhalis* and NTHi, where in the absence of *S. pneumoniae* culture, the proportion of NTHi and *M. catarrhalis* cultures were also reduced significantly. These clinical results support that of rodent models where established nasopharyngeal colonisation with *S. pneumoniae* promotes secondary *H. influenzae* colonisation. As such, the colonisation density was increased, of which *H. influenzae* showed an approximate 20% density increase compared to single *H. influenzae* nasopharyngeal colonisation (Margolis et al. 2010). Negative associations were evident among *S. aureus* and NTHi or *S. pneumoniae* cultures, where the presence of NTHi colonisation or *S. pneumoniae* colonisation was associated with a significant reduction in the proportion of *S. aureus* positive cultures. This latter relationship, however, was only significant in the distal nasopharynx. The observed bacterial co-colonisation relationships did not differ in COM prone children compared to non-COM prone children. These findings strengthen the clinical understanding of *S. pneumoniae* colonisation as a strong predictor for *M. catarrhalis* and NTHi colonisation, and negative *S. aureus* colonisation. This may therefore reflect synergistic mechanisms among their co-colonisation that are yet to be elucidated, indicating the need for further such investigations (Bogaert et al. 2004;

Regev-Yochay et al. 2004; Zemlickova et al. 2006; Pettigrew et al. 2008; Krishnamurthy et al. 2009).

The analysis of the significance of NPA otopathogen cultures as determinants of adenoid clinical cultures revealed that *S. pneumoniae, M. catarrhalis*, NTHi and *S. aureus* cultures in the NPA had strong positive correlations with the same species cultured from adenoid biopsies. The chi-square analyses revealed that if the specific bacterial species cultured negative or positive in the adenoid biopsies the proportion of the same bacterial species culturing negative or positive in the NPA respectively, was greater. With further statistical testing it was concluded that of the four principle otopathogens only *S. pneumoniae*, although not NTHi, *M. catarrhalis* or *S. aureus* NPA cultures, was an independent predictor of the equivalent species cultured from the adenoids. Furthermore, the likelihood of a child being diagnosed as COM prone, based on otopathogen screening in NPA cultures, was not significant. These results provide evidence for the benefit of clinicians not only utilising NPA cultures as a screening tool for colonisation of the greater nasopharynx, but also as a means of focusing antibiotic therapy for improving patient clinical outcomes.

The limitations of this study included the lack of comparison groups of children with an active infection, or healthy children without URTI or AH. These comparison groups would be restricted to NPA microbiology, due to the inability to remove adenoids from children with an active infection, or those without chronic URTI and/or AH diagnoses. When considering follow-on projects, the inclusion of such control groups would allow for the determination of bacterial nasopharyngeal changes that occur in pre-infection to infection states within children prone to COM and URTI in regional Queensland, enabling a more direct comparison with similar research conducted in Western Australia. Furthermore, it is recognised that employing PCR techniques may confirm the microbial results of the present study, but that an accurate reflection of the microbiology in the nasopharynx depends on more than simply screening methods used. By choosing intensive sampling techniques including adenoid biopsies and NPA, as opposed to swab techniques, the study design attempted to match a stringent sampling method with a clinically relevant screening tool, thereby enabling these results to be of direct relevance to general practitioners and to ENT specialists in applying evidence-based practices.

The sample size of this study was limited to 40 children and created difficulty in assessing demographic and environmental factors associated with clinical, microbiological and

lymphocyte measures. The power analysis to determine the sample size was based on changes in T_{reg} lymphocyte proportions of the adenoid between pneumococcal culture positive and negative children reported in literature. The significance reported in relation to these lymphocyte proportional changes with otopathogen culture are therefore supported with enough power to determine significance and have contributed to addressing the primary aims of this study, and advancing knowledge in this area. It is recognised that to confirm lack of, or significant relationships identified between demographic and environmental factors with clinical, microbiological and lymphocyte measures, the relationships will need to be explored in studies of larger cohorts, where the exploratory results here will provide a useful guide.

Mucosal and systemic humoral immunity to otopathogen colonisation and its association with cellular immunity is also important in understanding the aetiology of COM. The analysis of SIgA, PIgA and PIgG titres to pneumococcal sonicate antigen showed no differences between URTI and non-URTI children. Also no significant differences in the IgA antibodies were observed between COM and non-COM prone children. The PIgG levels were decreased in COM prone children compared to non-COM prone children, although the decline in antibody levels did not reach statistical significance. These trends were consistent with reports in previous studies (Hotomi 1999; Sharma et al. 2011). These same trends in pneumococcal-specific SIgA, PIgA and PIgG titres were also observed in *S. pneumoniae* culture positive children, compared to *S. pneumoniae* culture negative children, with results again comparable to previous reports (Zhang et al. 2006; Verhaegh et al. 2012). Therefore, such trends of decreased PIgG levels in *S. pneumoniae* culture negative children, and in COM prone children; trends that are supported in work by others showing statistical significance, may indicate that strong systemic humoral immunity plays a role in protection against pneumococcal colonisation, and a child's susceptibility to COM.

With respect to the pneumococcal-specific antibodies and dual colonisation with *S. pneumoniae* and *M. catarrhalis* or NTHi, the IgA levels in both saliva and plasma were decreased, compared to children with single *S. pneumoniae* colonisation. This contradicted a recent study by Xu & Pichichero (2014) in that enhanced antibody titres were observed in their pneumococcal-associated dual colonised children, compared to single *S. pneumoniae* colonised children. It is understood, however, that pneumococcal-specific antibody levels are dependent on the specific antigens used to detect the antibodies, so

perhaps such differences across the two studies may account for the variance in observed results.

The only significant association reported here in regards to the pneumococcal-specific antibody titres and lymphocytes, was the negative correlation of SIgA and $CD8^+$ lymphocytes in the blood. It is yet to be determined if this association is of importance, or if increases in circulating $CD8^+$ lymphocytes leads to a possible decline in locally produced mucosal antibody to pneumococcal nasopharyngeal colonisation. There are currently no published reports to compare this observation with.

Understanding changes in cellular and humoral lymphocyte profiles in the adenoids and blood of children is an important first step to determining how respiratory mucosal and systemic immunity integrate in children. The analysis of lymphocyte profiles from the adenoid and peripheral blood across the entire study population showed a significant decrease in B lymphocytes systemically, while a moderate increase in T and T_H lymphocytes was observed locally in the adenoids. This may indicate in children that local, cellular immunity and systemic, humoral immunity change in concert. Although B lymphocytes are the dominant subtype in the adenoids, the expansion of T and T_H lymphocytes here indicates an increased capacity locally for cellular immune activity. The T_{reg} lymphocytes showed direct proportional expansion in the nasopharynx and systemically that was significant, indicating that these immunosuppressive lymphocytes in the nasopharynx are tightly integrated, and reflected in the proportions of their circulating counterparts. In order to gain a comprehension of how closely such local and systemic relationships extend in relation to otopathogens, the immunosuppressive functionality of T_{reg} lymphocytes in the blood and adenoids needs to be measured against common otopathogens encountered in the nasopharynx. This has been investigated with pneumococcal antigen and T_{reg} lymphocyte associations and activity in the adenoids and blood from children, and more recently in murine models assessing the presence and function of T_{reg} lymphocytes in the MEM in response to NTHi-induced COM (Pido-Lopez et al. 2011; Zhang et al. 2011; Hirano et al. 2015; Jiang et al. 2015). Information is lacking though, in regards to other otopathogens, including *M. catarrhalis* and *S. aureus*.

With respect to COM specifically, in the blood and adenoids there were no observed differences between the lymphocyte subsets, including the T_{reg} population, in COM prone children compared to non-COM prone children, nor were any associations evident with the

lymphocyte subsets and COM. Rather, the results indicated that in children the proportion of lymphocyte subsets differed systemically with varying nasopharyngeal colonisation, as opposed to disease state. Circulating T_{reg} lymphocytes were found to increase significantly in children with positive nasopharyngeal culture, which was evident with both adenoid and NPA positive culture where blood-derived T_{reg} lymphocytes were increased. This provides evidence that increasing colonisation levels in the nasopharynx expand systemic T_{reg} lymphocytes. Furthermore, this confirms the hypothesis, in regards to systemic immunity but not locally in the adenoids, that commensal bacteria within the nasopharynx are positively associated with T_{reg} lymphocytes in the adenoids and blood of children with COM, thereby potentially contributing to host-tolerance of nasopharyngeal colonisation and the development of COM. The role played by these lymphocytes in a child's susceptibility to increased nasopharyngeal colonisation, and therefore increased risk of OM, is yet to be determined (Hallander et al. 1993; Pido-Lopez et al. 2011).

There is an understanding that T_{reg} lymphocytes are associated with *S. pneumoniae* positive culture, by suppressing pro-inflammatory responses to pneumococcal proteins. The published works though, vary in terms of whether such associations and activity occur in children or in late adolescence (Zhang et al. 2011; Palomares et al. 2012). To date, the only understanding of the role of regulatory lymphocytes in COM, is that performed by Hirano et al. (2015) where an NTHi-induced COM mouse model was shown to increase the percentage of T_{reg} lymphocytes in the MEM following COM with NTHi, compared to uninfected control mice. Furthermore, depletion of the T_{reg} lymphocytes improved NTHi clearance from the MEM and MEE, where pro-inflammatory cytokines were increased, compared to non-T_{reg} depleted control mice (Hirano et al. 2015).

In terms of specific otopathogen colonisation and lymphocyte proportional changes in the blood and adenoids, NTHi and *S. pneumoniae* carriage positive children had no significant changes in their lymphocyte profiles compared to NTHi and *S. pneumoniae* negative children. This supports the concept that NTHi and pneumococcal carriage influences adaptive immunity through active pathways rather than via polyclonal lymphocyte expansion. It is evident through various clinical studies and rodent models that *S. pneumoniae* colonisation induces active T_{reg} lymphocyte suppression of T_H and T_H17 responses in the nasopharynx, while NTHi-induced COM also triggers active T_{reg} lymphocyte suppressive responses in the MEM, thereby prolonging infection (Zhang et al. 2011; Palomares et al. 2012).

M. catarrhalis positive children had increased B lymphocytes in the blood compared to *M. catarrhalis* culture negative children; an observation that strengthens the concept that *M. catarrhalis* induces a systemic thymus-independent cellular response. This concept is supported by reports where human B lymphocyte proliferation to the MID protein was observed, which were capable of IgM, IgA and IgG secretions (Wingren et al. 2002). This is the first report of clinical *M. catarrhalis* carriage being associated with B lymphocyte expansion in children. *S. aureus* nasopharyngeal culture in children was associated with decreased T_C lymphocytes in the adenoids. The results presented here provide the first evidence of the association of nasopharyngeal *M. catarrhalis*, NTHi and *S. aureus* clinical culture with local and systemic lymphocyte subset profiles in children, however, the observations should be confirmed in a larger study cohort. The mechanisms of proportional changes in lymphocyte subsets in the blood and adenoid associated with nasopharyngeal culture are unknown and require further investigation. From the results presented here it evidently suggests that changes in lymphocyte subtype proportions are observed with microbial changes in the host rather than with clinical disease, this being particularly apparent in circulating T_{reg} and B lymphocyte increases with increased nasopharyngeal otopathogen and *M. catarrhalis* colonisation, respectively. It also demonstrates that changes in the host's nasopharyngeal flora reflect fluxes in the host's local and systemic lymphocyte profiles that are bacterial species-specific. With an aim to understand how otopathogens affect host immunity and the aetiology of disease, further investigation of otopathogen-induced changes to lymphocyte activity is warranted.

7.2 Summary of New Knowledge

This study adds to the existing body of knowledge of the pathophysiology of OM in Australian children, including collective data on the demography, trends in nasopharyngeal colonisation, pneumococcal-specific IgA and IgG titres, and lymphocyte subset profiles of the adenoid and blood in children prone to COM from regional Queensland. It involved the enrolment of 40 children from the Rockhampton district locale over a 12 month period, with data collection and clinical measurements of their demographic and clinical history, clinical microbiology from adenoid biopsies and NPA, antibody analysis from saliva and plasma samples, and phenotypic analysis of their adenoid and peripheral blood lymphocytes. This is the first substantial study of COM prone children in Queensland.

This research contributes to the understanding of the relationships of known demographic risk factors of OM with nasopharyngeal colonisation. The youngest child among siblings

has an increased risk of NTHi colonisation, although a decreased risk of *S. aureus* colonisation at the nasopharynx. ETS exposure increases the risk of nasopharyngeal colonisation with *M. catarrhalis* and *S. aureus*, and males have increased nasopharyngeal otopathogen colonisation. These results provide evidence for further investigation in a larger study cohort.

This research contributes to the understanding of the relationships of known demographic risk factors of OM with lymphocyte subset profiles of the adenoid and blood in children. New observations include that ETS exposure, birth order, household crowding, children with siblings having a history of OM, antibiotic or steroid therapy, and URTI (inclusive of tonsillitis and/or OM) do not change the proportion of the blood- or adenoid-derived B, T, T_C, T_H or T_{reg} lymphocytes in children, although these observations need confirmation in a considerably larger study. Increasing age is also not associated with changes in these lymphocyte subset proportions in the adenoid, or T and T_C lymphocytes proportions in the blood. The T_{reg} lymphocyte proportions in the blood however, do increase with age, and T_H blood lymphocytes are decreased in children who attend childcare. Such observations warrant their further investigation in a study of more participants, providing greater power to confirm these findings.

The nasopharyngeal colonisation trends observed in regional Queensland children prone to COM provides a greater understanding of the bacterial aetiology of OM in the region. It also contributes to a more comprehensive understanding of otopathogen carriage in Australasian children, when considered alongside carriage data from Western Australia, metropolitan Queensland and New Zealand. This clinical evidence relevant to regional Queensland children will assist clinicians in their approach to treatment of OM in such children. Therapeutic protocols should be aimed at reducing *S. pneumoniae* and *S. aureus* carriage in these children. Furthermore, the identification of microbial cultures of NPA samples as an indicative screening tool for *S. pneumoniae* growth in the greater nasopharynx provides evidence for physicians to use NPA samples as a diagnostic tool for nasopharyngeal pneumococcal carriage. This will further enable physicians to provide targeted antibiotic therapy to reduce *S. pneumoniae* carriage in COM prone children. This study provides strong evidence for investigating the potential of NPA cultures for predicting adenoid cultures in a larger study cohort, to confirm their promising clinical application for improved diagnostic practices.

The observations reported here for *S. pneumoniae*-specific antibodies in saliva and plasma from COM prone and non-COM prone children, with and without nasopharyngeal otopathogen culture, and the associations with lymphocytes from the adenoids and blood, provides knowledge for further understanding humoral immunity in pneumococcal colonisation and COM. This investigation led to new findings in relation to the correlations between *S. pneumoniae*-specific antibodies and lymphocyte subsets in the adenoids and blood from children. No significant correlations were identified in B, T, T_C, T_H or T_{reg} lymphocytes in the adenoids, or those of the blood, except for T_C lymphocytes. SIgA correlated negatively with T_C lymphocytes in the blood. It remains unclear if this relationship is of importance in colonisation by *S. pneumoniae*. Furthermore, these results support their investigation in a larger study, to confirm the existing, or lack of existing relationships.

This study contributes to a greater understanding of adenoid and blood lymphocyte profiles in COM prone children, for which no observed differences between the lymphocyte subsets were evident, compared to non-COM prone children. This is the first report regarding adenoid and blood T_{reg} lymphocyte populations in COM prone children. This provides evidence that changes in lymphocyte function rather than proportions need to be investigated. In supporting the concept of functionality as a key point of interest, adenoid and blood lymphocyte subset proportions differed with antigen exposure associated with varying nasopharyngeal colonisation in children, as opposed to disease state. This is the first report of lymphocyte proportional changes with general nasopharyngeal otopathogen culture, *M. catarrhalis*, *S. aureus* and NTHi culture in children; circulating T_{reg} lymphocytes are significantly increased with positive nasopharyngeal culture. *M. catarrhalis* carriage is associated with systemic B lymphocyte expansion, and *S. aureus* carriage is associated with decreased T_C lymphocytes in the adenoids. NTHi colonisation did not change lymphocyte subset profiles in the adenoids or blood. These results provide clear evidence for further investigation in a larger study to confirm these relationships and potentially explore associated functional aspects between colonisation and lymphocyte subsets.

7.3 Future Work

In order to confirm age-associated systemic increases in T_{reg} lymphocyte proportions, a larger scale study investigating this is needed. Furthermore, in order to determine if such T_{reg} lymphocyte proportions are associated with immune tolerance to otopathogens, thereby

reducing ear disease rates in adulthood, future functional studies are also needed. Such studies should investigate the T_{reg} lymphocyte suppressive activity induced by otopathogen nasopharyngeal carriage, and whether or not such functionality differs in children and adults with and without OM. In order to confirm that communal childcare is associated with a decrease in circulating T_H lymphocytes, the relationship should be explored in a larger study cohort. Microbial dissemination and functional aspects of cellular immunity may also need to be measured and compared in children who do and do not attend childcare. This will provide a greater understanding of how a communal environment for children affects their cellular immunity and its functional activity against encountered otopathogens.

This study has highlighted the clinical importance of further investigation into elucidating the synergistic mechanisms involved in the co-colonisation of *S. pneumoniae* with *M. catarrhalis* and NTHi, and into the negative trends with *S. aureus* nasopharyngeal colonisation. This is particularly important, as *S. pneumoniae* was identified as the leading otopathogen in COM prone children in regional Queensland, and, therefore, it is necessary to understand how it promotes co-colonisation and increased carriage density, as these are factors that increase the risk of OM (Jacoby et al. 2011). Furthermore, it is of clinical relevance to determine the distribution of pneumococcal carriage flora that are vaccine and non-vaccine serotypes. Such data could be analysed against vaccine compliance in order to determine how the pneumococcal immunisation regimes influences pneumococcal carriage in COM prone children of regional Queensland. In order to verify the preferred screening method for *S. pneumoniae* carriage, further larger studies should consider the inclusion of repeated measure approaches to determine the predictive diagnostic value, and the precision of each screening method under evaluation.

Herein it is shown that T_{reg} lymphocyte profiles in the adenoids are reflected in the blood, and that T_{reg} lymphocytes expand systemically with positive otopathogen colonisation in the nasopharynx. In order to gain an understanding of how closely this local and systemic relationship extends in relation to otopathogens, the immunosuppressive functionality of T_{reg} lymphocytes in the blood and adenoids needs to be measured against common otopathogens encountered in the nasopharynx, including investigations into antigen-specific responses. Such clinical research is lacking in relation to NTHi, *M. catarrhalis* and *S. aureus*. This is necessary to determine the role of these lymphocytes in a child's susceptibility to increased nasopharyngeal colonisation, and therefore increased risk of OM. This study has highlighted that changes to lymphocyte profiles in the blood and

adenoids in relation to otopathogens in the nasopharynx is species-specific. This provides a justification to support further research into the mechanisms by which the otopathogens induce such proportional changes in lymphocyte subsets in the blood and adenoids. Furthermore, investigation into otopathogen-induced changes to lymphocyte activity would improve the understanding of how otopathogens affect host immunity and the aetiology of disease.

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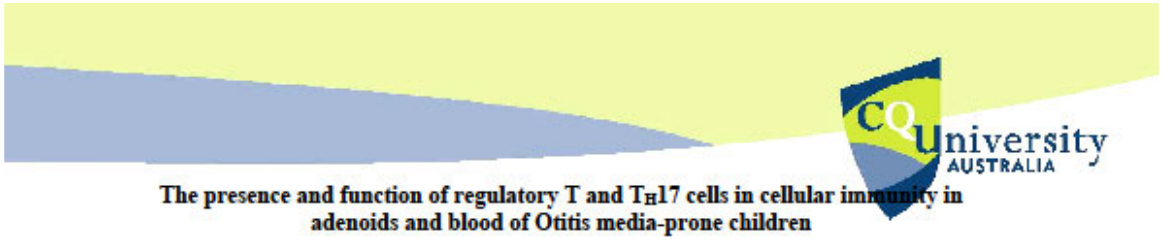
9 APPENDICES

9.1 Appendix A



The presence and function of regulatory T and T_H17 cells in cellular immunity in adenoids and blood of Otitis media-prone children

PARTICIPANT INFORMATION AND CONFIDENTIAL QUESTIONNAIRE

Participant laboratory code: _____ Date of collection: _____

Residential postcode of Child: _____ Child's age (years): _____

Child's Gender: Male ☐ Female ☐

This child is of Aboriginal or Torres Strait Islander heritage: Yes ☐ No ☐

Tissues collected:

Adenoids	☐	Saliva	☐
Tonsils	☐	Nasal secretion	☐
Blood	☐		

Brief clinical history leading to reason for removal of tissue: _____

_____

_____

Parent or Legal Guardian: Please take a few minutes to complete these questions. All the questions below pertain to the background component of this research.

1. **How many children under the age of 15 live in the house with this child?**

None	1	2	more than 2	Unknown
☐	☐	☐	☐	☐

2. **If you answered 1 or more:**

a. is this child the:

Eldest	Middle	Youngest	Unknown
☐	☐	☐	☐

b. have the other children had middle ear infections?

No	Unknown			
☐	☐			
Yes	→	Less than 3 times	☐	
☐		3 or more times	☐	

3. **Do any people who regularly live in the same household with this child smoke?**

No	Unknown		
☐	☐		
Yes	→	Inside the house	☐
☐		Outside the house	☐

4. **Is this child regularly (at least weekly) exposed to cigarette smoke at another location?**

Yes	No	Unknown
☐	☐	☐

5. **Does this child attend a day care centre, kindergarten, preschool or school?**

Yes	No	Unknown
☐	☐	☐

6. **Has this child had their routine immunisations?**

Yes	No	Some	Unknown
☐	☐	☐	☐

Thank you for your participation in this short questionnaire.

9.2 Appendix B

The presence and function of regulatory T and T_H17 cells in cellular immunity in adenoids and blood of Otitis media-prone children

CASE RECORD FORM ENROLEMENT

NOTE: A SIGNED CONSENT FORM IS REQUIRED BEFORE ENROLMENT CAN BE PROCESSED
All information documented in this form is strictly confidential and will be managed in accordance with the research ethics approval for this project. After informed voluntary consent is obtained from the parent / guardian of the patient, the parent / guardian is to complete the 1 page questionnaire and may seek assistance from the doctor or clinical staff with the doctor, in completing this. Information from the questionnaire will be documented herein by the doctor. This form will be completed by the doctor and it will include relevant information obtained from the patients' medical record.

PROCEDURE FOR COMPLETING THE CASE RECORD FORM

This Case Record Form is part of the general enrolment process and consists of 3 parts:
Part A Information on Patient details and enrolment specifics.
Part B Information included from participant questionnaire.
Part C Information included from participant's relevant patient medical records.

PART A

Patient's Details			
Name:	D.O.B:	Male ☐	Female ☐
Address:			
Phone:	Email:		
Medicare Number:			
Name of Parent / guardian of patient:			
Enrolment Specifics			
Project ethics approval number:			
Date of enrolment:	UR Number:		
Enrolling Doctor:		GP ☐	Specialist ☐
Doctor Address:			
Date of withdrawal from study (if requested):		Withdrawn ☐	
Doctor processing withdrawal:		GP ☐	Specialist ☐
Doctor Address:			

PART B

Informed Consent					
A signed informed consent for participation has been obtained				**Yes** ☐	
Participant Questionnaire Details					
Age:	Post code:			PL Code:	
This child is of Aboriginal and / or Torres Strait Islander (ATSI) heritage:				Yes ☐	No ☐
Tissues collected:	Adenoids ☐	Tonsils ☐	Blood ☐	Saliva ☐	Nasal secretions ☐

How many children under the age of 15 live in the house with this child?

None ☐ 1 ☐ 2 ☐ more than 2 ☐ Unknown ☐

If participant answered 1 or more:

a) is this child the:

Eldest ☐ Middle ☐

Youngest ☐ Unknown ☐

b) have the other children had middle ear infections?

No ☐ Unknown ☐

Yes ☐ → Less than 3 time ☐
3 or more times ☐

Do any people who regularly live in the same household with the participant smoke?

No ☐ Unknown ☐

Yes ☐ → Inside the house ☐
Outside the house ☐

Is the participant regularly (at least weekly) exposed to cigarette smoke at another location?

Yes ☐ No ☐ Unknown ☐

Does this participant attend a day care centre, kindergarten, preschool or school?

Yes ☐ No ☐ Unknown ☐

Has the participant indicated that they have had their routine immunisations?

Yes ☐ No ☐ Partial ☐ Unknown ☐

PART C

Participant's Relevant Clinical Details
Date of last visit:
Is the participant prone to OM? Yes ☐ No ☐
Based on the information provided herein and your professional opinion, please assign the participant to either: Group 1 - Non-OM prone ☐ Group 2 - OM prone ☐

Does the participant suffer with an Otitis media (OM) condition? Yes ☐ No ☐

If Yes, detail the specific OM diagnosis:

If Yes, has this participant suffered with OM (acute, chronic or recurrent episodes) for:
Less than 3 months ☐ More than 3 months, however less than 6 months ☐
More than 6 months ☐

If Yes, has this participant also suffered with other respiratory infections? Yes ☐ No ☐

Provide details of the specific respiratory infection diagnosis:

If the participant does not have OM, does the participant suffer with a respiratory infection condition? Yes ☐ No ☐

If Yes, detail the specific respiratory infection diagnosis:

If Yes, has this participant suffered with respiratory infections (acute, chronic or recurrent episodes) for: Less than 3 months ☐ More than 3 months, however less than 6 months ☐
More than 6 months ☐

Does the participant suffer from enlarged adenoids or enlarged tonsils? Yes ☐ No ☐

Is the participant undergoing adenoidectomy ☐ / tonsillectomy ☐ for reasons unrelated to OM?

Yes ☐ No ☐

if Yes, please state reason:

Is the participant undergoing adenoidectomy ☐ / tonsillectomy ☐ for reasons related to OM?

Yes ☐ No ☐

Has the participant had their routine immunisations?

Yes ☐ No ☐ Partial ☐ Unknown ☐

If Yes, have they received any of the licensed pneumococcal vaccines (Prevenar®, Prevenar13®, Pneumovax 23®)? Yes ☐ No ☐ Partial ☐ Unknown ☐

Has the participant had antibiotic therapy for respiratory infections in the last 6 months?

Yes ☐ No ☐

If Yes, please provide details of medications prescribed (drug name sufficient and details of course duration – regular 7 day treatment with repeat, or ongoing):

Has the participant had any steroid medications prescribed (intranasal, inhaled or oral) for the treatment and / or management of respiratory infections in the last 6 months?

Yes ☐ No ☐

If Yes, please provide details of medications prescribed (drug name sufficient):

Please describe any surgical intervention strategies the participant has received in the past to treat or manage respiratory infections:

Clinical Microbiology Tests

Nasopharyngeal aspirates cultured for:

S. pneumoniae	☐	*M. catarrhalis*	☐	non-typeable *H. influenzae*	☐
S. aureas	☐	*S. pyrogenes*	☐	Group A streptococcus	☐
A. otitidis	☐	*P. aeruginosa*	☐		

Culture Results:

S. pneumoniae	positive	☐	negative	☐
M. catarrhalis	positive	☐	negative	☐
non-typeable *H. influenzae*	positive	☐	negative	☐
S. aureas	positive	☐	negative	☐
S. pyrogenes	positive	☐	negative	☐
Group A streptococcus	positive	☐	negative	☐
A. otitidis	positive	☐	negative	☐
P. aeruginosa	positive	☐	negative	☐
Other	positive	☐	organism name:	

Declarations and Signatures

Name of person making the report:

Signature: Date:

Relationship to the project (if not the principle investigator):

Principle Investigator for research and site:

Signature: Date:

Details herein from Part B and Part C ONLY are to be transferred to the researchers at CQUniversity for data collation, processing, analysis and interpretation in the study.

9.3 Appendix C

The presence and function of regulatory T and T_H17 cells in cellular immunity in adenoids and blood of Otitis media-prone children

Informed Consent Form for Collection of Samples and Clinical Information for Research

I consent voluntarily as the parent or legal guardian of my child less than 12 years of age to participate in this research and agree that:

1. An Information Sheet has been provided to me that I have read and understood;

2. All questions I had about the research have been answered to my satisfaction by the Information Sheet and any further explanation required has been provided verbally;

3. As my child is young (between 2 and 7 years of age) I appreciate that they may have difficulty in understanding the complexity of this research. Due to this I understand that I am authorised to provide consent for my child's participation in the research on my child's behalf.

4. I understand that I have the right to withdraw my child's samples from this research at any time without penalty, and that such samples would be destroyed accordingly. I understand that my child's data must remain in the study database in order for the study to be scientifically valid;

5. I understand the research findings will be included in the researcher's publication(s) such as conferences, scientific publications and other methods of publication stated in the Information Sheet;

6. I understand how the study will preserve anonymity and maintain confidentiality of participants;

7. I am aware that a Plain English statement of results will be available on the web address provided in the Information Sheet;

8. I agree that I am providing informed consent for the collection of my child's tissues, samples and/or specified re-identifiable general and clinical information to participate in this PhD research. I am consenting to the collection of the following samples and information to be used for the purposes specific to this PhD research (please tick the boxes next to the tissues, clinical information and Participant Questionnaire that you wish to give your consent for to be used in this research):

Adenoids	☐	Saliva	☐	Participant Questionnaire	☐
Nasal secretion	☐	Blood	☐	Clinical Information	☐
Tonsils	☐				

9. I understand for samples and information that I do not agree to be donated to this research will be destroyed appropriately.

Signature of parent or guardian: _____ Date: _____

Name of parent or guardian (please print): _____

Name of participant (child 2 to 7 years of age): _____

Where relevant to the research project, please check the box below:

	YES	NO
1. I wish to have a Plain English statement of results posted to me at the address I provide below.		

Postal Address: _____

E-mail Address: _____

CQUHREC clearance number: _____ Version 5.0 16/01/2013

9.4 Appendix D

Table 9.1 Univariate logistic regression for demographic & environmental factors associated with COM

Risk Factor	**COM (n=20) OR (95% CI)**	***p* value**
Age (2 - 3 years reference)**,** 4 - 7 years	1.22 (0.35 – 4.24)	0.75
Sex Male	0.81 (0.22 – 2.91)	0.74
Number of children in household ≤15 years of age (≤ 2 children reference), ≥3 children	1.23 (0.35 – 4.31)	0.75
Child's birth order (Eldest reference)		
Middle	0.36 (0.06 – 2.08)	0.25
Youngest	0.47 (0.11 – 1.92)	0.47
Siblings with a history of OM	1.00 (0.29 – 3.48)	1.00
ETS exposure	0.46 (0.11 – 1.94)	0.29
Childcare	0.71 (0.14 – 3.66)	0.68
History of URTI	2.67 (0.65 – 10.97)	0.17
Antibiotic therapy within the last 6 months	10.23 (1.21 – 93.34)	0.04
Steroid therapy within the last 6 months	0.63 (0.09 – 4.24)	0.64

AH = adenoid hypertrophy; CI = confidence interval; COM = chronic otitis media; ETS = environmental tobacco smoke; OM = otitis media; OR = odds ratio; URTI = upper respiratory tract infection.

Table 9.2 Independent student t-test for differences in adenoid lymphocyte subset percentages with demographic factors

	Adenoid derived lymphocytes				
	B Lymphocytes	**T Lymphocytes**	**T_C lymphocytes**	**T_H lymphocytes**	**T_{reg} lymphocytes**
Risk Factor	***M*%, *SD*%, *p* values**	***M*%, *SD*%, *p* value**	***M*%, *SD*%, *p* value**	***M*%, *SD*%, *p* value**	***M*%, *SD*%, *p* value**
Age (continuous)					
2 - 3 years (*n* = 20)	53.0, 8.5	39.5, 10.3	7.1, 2.5	28.6, 7.3	3.9, 1.0
4 - 7 years (*n* = 18)	53.0, 7.4, 0.99	40.7, 7.6, 0.68	6.6, 2.7, 0.57	28.6, 5.8, 0.98	4.3, 1.3, 0.31
Sex					
Male (*n* = 24)	52.6, 8.4	40.5, 9.8	7.2, 2.7	29.5, 7.5	4.1, 1.1
Female (*n* = 14)	53.5, 7.1, 0.74	39.3, 7.6, 0.69	6.4, 2.4, 0.36	27.1, 4.3, 0.30	4.1, 1.3, 0.95
Number of children in household ≤15 years of age					
≤ 2 children (*n* = 21)	53.2, 5.4	40.8, 6.9	7.1, 2.3	29.6, 5.4	4.4, 1.2
≥3 children (*n* = 17)	52.7, 10.3, 0.86	39.2, 11.2, 0.60	6.6, 2.9, 0.50	27.4, 7.7, 0.32	3.8, 1.1, 0.17
Child's birth order					
Youngest (*n* = 14)	53.1, 9.2	41.3, 9.4	7.1, 2.7	29.5, 7.8	4.1, 0.8
Middle (*n* = 8)	51.7, 7.8	41.8, 6.6	7.6, 2.5	28.3, 4.0	4.2, 1.3
Eldest (*n* = 16)	53.5, 7.0, 0.87	38.1, 9.8, 0.53	6.3, 2.6, 0.46	28.0, 6.6, 0.81	4.0, 1.4, 0.88
Siblings with a history of OM					
Yes (*n* = 17)	50.3, 9.1	**44.1, 8.5**	**7.9, 2.5**	30.5, 6.6	4.0, 1.3
No (*n* = 21)	55.1, 6.1, 0.06	**36.8, 8.2, 0.01**	**6.1, 2.4, 0.03**	27.1, 6.2, 0.12	4.3, 1.1, 0.51
ETS exposure					
Yes (*n* = 11)	53.3, 6.6	37.7, 9.8	6.5, 2.4	27.4, 5.6	4.2, 0.8
No (*n* = 27)	52.8, 8.4, 0.86	41.0, 8.6, 0.31	7.0, 2.6, 0.61	29.1, 5.9, 0.46	4.1, 1.3, 0.83
Childcare					
Yes (*n* = 31)	53.2, 7.3	39.6, 8.7	6.8, 2.7	28.0, 5.9	4.2, 1.2
No (*n* = 7)	52.0, 10.6, 0.73	42.1, 10.5, 0.50	7.3, 2.3, 0.61	31.7, 8.5, 0.17	3.7, 1.2, 0.32
History of URTI					
Yes (*n* = 26)	54.1, 7.7	40.1, 8.3	6.9, 2.7	29.5, 5.8	4.1, 1.1
No (*n* = 12)	50.5, 7.9, 0.18	39.9, 10.8, 0.93	6.9, 2.6, 0.95	26.7, 7.9, 0.24	4.2, 1.4, 0.71

ETS = environmental tobacco smoke; *M* = mean; OM = otitis media; *SD* = standard deviation; T_C = cytotoxic T lymphocyte; T_H = T helper lymphocyte; T_{reg} = regulatory T lymphocyte; URTI = upper respiratory tract infection. Values in bold indicate significance.

Table 9.3 Binary logistic regression odds ratios & 95% confidence intervals for species-specific colonisation predicting specific otopathogen colonisation at the adenoid & NPA

Adenoid positive culture								
	S. pneumoniae		***M. catarrhalis***		***NTHi***		***S. aureus***	
Adenoid positive culture	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**
S. pneumoniae			**8.21 (1.30 – 52.00), 0.03**	ns	**4.43 (1.02 – 19.27), 0.047**	**6.89 (1.00 – 47.47), 0.050**	0.16 (0.02 – 1.46), 0.11	NA
M. catarrhalis	**8.21 (1.30 – 52.00), 0.03**	**6.9 (1.01 – 47.32), 0.049**			3.11 (0.58 – 16.83), 0.19	NA	0.00 (0.00 - 0.00), 0.99	NA
NTHi	**4.43 (1.02 – 19.27), 0.047**	3.72 (0.77 – 17.98), 0.10	3.11 (0.60 – 16.83), 0.19	NA			0.00 (0.00 - 0.00), 0.99	NA
S. aureus	0.16 (0.02 – 1.46), 0.11	NA	0.00 (0.00 - 0.00), 0.99	NA	0.00 (0.00 - 0.00), 0.99	NA		
NPA positive culture								
	S. pneumoniae		***M. catarrhalis***		***NTHi***		***S. aureus***	
NPA positive culture	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**	**Univariate *n* = 37, OR (95% CI), *p* value**	**Multivariate *n* = 37, OR (95% CI), *p* value**
S. pneumoniae			**7.67 (1.36 – 43.14), 0.02**	ns	**16.67 (1.57 – 177.49), 0.02**	**14.16 (1.28 – 156.38), 0.03**	**0.16 (0.02 – 1.46), 0.11**	NA
M. catarrhalis	**7.67 (1.36 – 43.14), 0.02**	**15.02 (1.91 – 118.42), 0.01**			0.86 (0.08 – 8.97), 0.90	NA	0.19 (0.02 – 1.76), 0.14	NA
NTHi	**16.67 (1.57 – 177.49), 0.02**	**34.34 (2.42 – 487.53), 0.009**	0.86 (0.08 – 8.97), 0.90	NA			0.00 (.00 - .00), 0.99	NA
S. aureus	0.00 (0.00 – 0.00), 0.99	NA	0.19 (0.02 – 1.76), 0.14	NA	0.00 (.00 - .00), 0.99	NA		

CI = confidence interval; NA = not applicable; ns = non-significant; NTHi = non-typeable *H. influenzae*; OR = odds ratio. Only variable with a *p* value of 0.10 or less were included in a multivariate logistic regression analysis.

Table 9.4 Binary logistic regression odds ratios & 95% confidence intervals for colonisation predicting COM proneness

	COM proneness	
Positive culture	**Univariate *n* = 20, OR (95% CI), *p* value**	**Multivariate *n* = 20, OR (95% CI), *p* value**
Nasopharyneal culture	0.26 (0.02 – 3.14), 0.29	NA
Nasopharyngeal multiple culture	1.28 (0.33 – 5.04), 0.72	NA
Adenoid culture	0.88 (0.17 – 4.70), 0.88	NA
NPA culture	0.49 (0.10 – 2.41), 0.40	NA
***S. pneumoniae* culture**	2.04 (0.48 – 8.75), 0.34	NA
***M. catarrhalis* culture**	0.85 (0.17 – 4.20), 0.84	NA
NTHi culture	0.25 (0.05 – 1.14), 0.07	0.22 (0.01 – 6.05), 0.37
***S. aureus* culture**	0.96 (0.23 – 3.91), 0.95	NA

CI = confidence interval; NTHi = non-typeable *H. 160nfluenza*; OR = odds ratio. Only variable with a *p* value of 0.10 or less were included in the multivariate logistic regression analysis. Nasopharyngeal otopathogen culture is inclusive of both adenoid and NPA cultures, where at least one otopathogen is culture positive. Nasopharyngeal multiple culture is inclusive of both adenoid and NPA cultures, where two or more otopathogens are culture positive. Adenoid culture is where any otopathogen has cultured positive from the adenoid biopsy. NPA culture is where any otopathogen has cultured positive from the NPA sample. Species-specific cultures are inclusive of adenoid and NPA positive culture, relevant to the specific species listed.

Table 9.5 Spearman's rho correlation of blood & adenoid lymphocyte subsets

Blood lymphocytes	Spearman rho correlation	Adenoid Lymphocytes				
		B lymphocytes	**T lymphocytes**	**T_H lymphocytes**	**T_C lymphocytes**	**T_{reg} lymphocytes**
B lymphocytes	Correlation Coefficient	0.22	**-0.40**	-0.22	**-.037**	0.06
	p value	0.19	**0.013**	0.18	**0.02**	0.75
	n	38	**38**	38	**38**	35
T lymphocytes	Correlation Coefficient	0.10	0.02	0.02	0.16	-0.02
	p value	0.55	0.89	0.91	0.34	0.91
	n	38	38	38	38	35
T_C lymphocytes	Correlation Coefficient	-0.01	-0.03	0.22	0.08	0.12
	p value	0.96	0.86	0.18	0.65	0.50
	n	38	38	38	38	35
T_H lymphocytes	Correlation Coefficient	-.04	0.15	0.08	0.25	-0.06
	p value	0.81	0.38	0.62	0.13	0.74
	n	38	38	38	38	35
Treg lymphocytes	Correlation Coefficient	0.15	-0.02	0.01	0.06	**0.62**
	p value	0.36	0.93	0.95	0.70	**0.000**
	n	38	38	38	38	**35**

T_C = cytotoxic T lymphocyte; T_H = T helper lymphocyte; T_{reg} = regulatory T lymphocyte. Values in bold indicate significance.

Table 9.6 Spearman's rho correlation of SIgA, PIgA & PIgG & lymphocyte subsets from the blood & adenoids

Pneumococcal-specific Immunoglobulin	Spearman rho correlation	Blood Lymphocytes				
		B lymphocytes	**T lymphocytes**	**T_C lymphocytes**	**T_H lymphocytes**	**T_{reg} lymphocytes**
SIgA	Correlation Coefficient	-0.24	-0.16	**-0.40**	0.05	0.06
	p value	0.16	0.34	**0.02**	0.76	0.74
	n	36	36	**36**	36	36
PIgA	Correlation Coefficient	-0.26	0.09	-0.11	0.08	0.06
	p value	0.11	0.59	0.50	0.64	0.71
	n	39	39	39	39	39
PIgG	Correlation Coefficient	0.04	0.09	0.21	-0.04	0.04
	p value	0.80	0.59	0.20	0.83	0.80
	n	39	39	39	39	39
		Adenoid Lymphocytes				
SIgA	Correlation Coefficient	-0.22	0.30	0.06	0.17	0.06
	p value	0.20	0.08	0.71	0.34	0.76
	n	35	35	35	35	32
PIgA	Correlation Coefficient	-0.20	0.30	0.12	0.18	-0.03
	p value	0.22	0.06	0.48	0.28	0.85
	n	38	38	38	38	35
PIgG	Correlation Coefficient	-0.19	0.13	0.05	0.09	-0.05
	p value	0.26	0.45	0.76	0.59	0.80
	n	38	38	38	38	35

PIgA = plasma immunoglobulin A; PIgG = plasma immunoglobulin G; SIgA = salivary immunoglobulin A; T_C = cytotoxic T lymphocyte; T_H = T helper lymphocyte; T_{reg} = regulatory T lymphocyte. Values in bold indicate significance.